Development of DNA fingerprints of *Dichanthium* genotypes and their association with stress responsive characters

THESIS
SUBMITTED TO

Bundelkhand University Jhansi (U.P.)

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
(BIOTECHNOLOGY)

By

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Under the supervision of

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2007

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ग्वालियर मार्ग, झाँसी – 284 003 (उ.प्र.) भारत
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The Vice Chancellor
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Sub: Submission of Ph.D. Thesis

Sir,

I am forwarding herewith the thesis entitled "Development of DNA fingerprints of *Dichanthium* genotypes and their association with stress responsive characters" by Mr. Raghvendra Saxena for the degree of Doctor of Philosophy in Biotechnology, Bundelkhand University, Jhansi. The work has been carried out at Indian Grassland and Fodder Research Institute, Jhansi under the supervision of Dr. Amaresh Chandra.

Thanking you

Yours faithfully

(K. A. Singh)

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CERTIFICATE

It is certified that the thesis entitled "Development of DNA fingerprints of *Dichanthium* genotypes and their association with stress responsive characters" is an original piece of work done by Mr. Raghvendra Saxena under my supervision and guidance for the degree of Doctor of Philosophy in Biotechnology, Bundelkhand University, Jhansi.

I further certify that:

- It embodies the original work of candidate himself.
- It is up to the required standard both in respect of its contents and literary presentation for being referred to the examiners.
- The candidate has worked under me for the required period at Indian Grassland and Fodder Research Institute, Jhansi.
- The candidate has put in the required attendance in the department during the period.
- No part of the thesis has been submitted for any other degree or diploma.

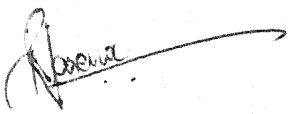
19/5/07

Dr. Amaresh Chandra

(Supervisor)

DECLARATION

I hereby declare that the thesis entitled "Development of DNA fingerprints of *Dichanthium* genotypes and their association with stress responsive characters" being submitted for the degree of Doctor of Philosophy in Biotechnology, Bundelkhand University, Jhansi (UP) is an original piece of research work done by me under the supervision of Dr. Amaresh Chandra, IGFRI, Jhansi and to the best of my knowledge, any part or whole of this thesis has not been submitted for a degree or any other qualification of any University or examining body in India / elsewhere.



(Raghvendra Saxena)

Acknowledgements

I consider it a privilege to express my deep sense of gratitude, respect and indebtedness to my supervisor Dr. Amaresh Chandra., Senior Scientist (Biochemistry),C.I. Division IGFRI Jhansi for constant encouragement, erudite guidance and constructive criticism during the course of present investigation. He has nurtured my capabilities and always given me full freedom to carryout my research work.

I offer my esteemed thanks to Dr. O.P.S. Verma, Dr. A.K. Roy, Dr. M.J. Baig, Dr. Anjali Anand for their suggestions and helping attitude throughout the tenure of my research work.

My heartfelt thanks are due to Dr. S. N. Zadoo, Dr. R.N. Choubey, (Former Head) and Dr G. P. Shukla, Head, Crop Improvement Division, who have extended their full support and guidance.

I am grateful to Dr. K. A. Singh, Director IGFRI, Jhansi, for his valuable suggestions and encouragement. I am also highly thankful to Dr P. S. Pathak, the then Director IGFRI for granting permission and providing necessary help and guidance.

My sincere thanks are also due to all scientists of Crop Improvement Division and other divisions who have helped many times. Particular thanks are due to Dr. D.R Malviya and Dr. Pankaj Kaushal for their cooperation and allowing me to carry out certain work in their laboratory.

I wish to acknowledge my thanks to my colleagues Dr. Bijender, Dr Kuldeep Diwedi, Dr. Harishankar, Dr. Ashwani Mishra Dr. Archna Dubey and Dr. Niti Kushwah, who have extended all co-operations throughout the working period.

I also wish to extend my thanks to my labmates, Miss Sanchita tiwari, Deepti Nagaich, Poornima Swami and Mr. Kapil Tiwari,for their unconditional support, always ready to help gesture, keeping lively and constructive ambience of the lab.

I further extend my heartfelt acknowledgement to Aarti Khare, Aradhana Yadav, Rupali Tiwari, Bilal Ahmed, Ambika Khare, Shalini Pathak, Ankit Agarawal and Sahid Ali Siddiqui.

I also acknowledge the help and support provided by Mr Harish Chandra Pandey throughout my research work.

It is my pleasure to acknowledge the help received from Mr. Shailendra Sinha, Mr Dhiraj Dhingra, Mr. Md. Irfan and Mr. Mahesh Jetwani who always extended their full support to me at any time.

I also acknowledge my school days friends Rahul Dubey and Dhirendra for their constant encouragement unshaken support all the time.

Lastly, this acknowledgement would be incomplete without expressing my indebtedness to my family members who have been a constant source of inspiration and support all through my life. I am at a loss of words towards my loving parents whose unconditional love and support always inspired me to touch new heights. I further express my deep sense of respect towards my elder brother Arvind who always remains a source of inspiration and motivation for me. I express my sincere gratitude and love to my younger brother Vikrant and sister Trupti for their infinite affection and bondless love.

At this time, it is my great pleasure to thanks my wife Pratiksha for showing deep faith in me, and who has always remains a source of inspiration and encouragement in this endeavor.

I acknowledge my all friends and well wishers whom I may not have mentioned here but have been always helpful at appropriate time.

Finally, Council of Scientific and Industrial Research is greatly acknowledged for providing financial assistance (JRF/SRF) throughout the tenure of work.

Date: 19/05/07

(RAGHVENDRA SAXENA)

Contents

Introduction

Plant genetic resources (PGR) constitute the basic raw material essentially required for the genetic improvement programme. Collection, evaluation, documentation and conservation are the four main pillars of PGR. Proper evaluation and use of plant genetic resources are essential for future agricultural progress especially when a number of precious germplasm are declining with time. There is an urgent need to maintain the germplasm and efforts should be made to organize research programme on germplasm characterization and its efficient utilization.

Grasses belongs to the family poaceae (*Gramineae*), which is the fourth largest family. They are grouped into 650 to 785 genera containing about 10,000 species (Watson and Dallwitz, 1992). All the cereal crops and 75% of cultivated forage species are grasses (Nelson and Moser, 1995). Although the grass family is large, relatively few species have been developed as forage crops. It is generally believed that grasses have a wider range of adaptation to temperature and rain fall than any other family of flowering plants. The cultivated grasses contain some well adapted, productive forage species that are distributed worldwide. The most intensively used forage and turf grasses include fescues (*Festuca* spp.), rye grass (*Lolium* spp.), bent grass (*Agrostis* spp.), blue grass (P*oa* spp.), broom grass (*Bromus* spp.), orchard grass (*Dactylic* spp.), bermuda grass (*Cynodon* spp.) and *Panicum* spp.

On world wide basis, grassland acerage is estimated to be twice that of cropland (Jauhar, 1993). Forage grasses are often grown on land and soils that are less suitable for cultivation of crops used directly for human consumption. Forage grasses are most widely grown but least appreciated, commodity. When consideration is given to their direct and indirect benefits, it is obvious that research and development in this agriculture sector has been neglected to a large

extant. In contrast to cash crops, the cash value of forage is realized through animals.

Marvel grass (*Dichanthium annulatum* Forsk.) is an important perennial range grass of tropical and subtropical regions. In India, it is a constituent of two major grass covers i.e., *Dichanthium-Cenchrus-Lasiurus* and *Sehima-Dichanthium* (Dabadghao and Shankarnarayan, 1973). Though highest green biomass is obtained in monsoon season, it persists and survives under harsh and dry environmental conditions. To date around 20 species of the genus have been reported, 8 of these found in India in various agro-ecological zones (Arora *et al.*, 1975). Two species viz. *D. annulatum* and *D. caricosum* are widely used for forage production because of their high green biomass and persistence in harsh environmental conditions. Being indigenous to the Indian as well as African gene centers, the genus shows remarkable genetic diversity in India and South Africa (Mehra and Magoon, 1974). Marvel grass has wide range of adaptations ranging from low rainfall areas in Rajasthan and Gujarat to heavy rainfall areas of Western and Southern India. The basic chromosome number in *Dichanthium* species is ten but largely exist in different ploidy levels ($2n$ = 2x = 20, 4X= 40 and 6X= 60) with distinct morphological characters (Mehra, 1961; Fedorov, 1974). Of these, tetraploids are most common. Though IGFRI maintained sufficient number of accessions collected from different parts of the country under plant genetic resources program, the study pertaining to cytological aberration which might be existing due to their growth in undulated, wild and extreme environment conditions is lacking. Since they grow in extreme environmental conditions, the biochemical and histological changes caused by moisture stress and their study may lead to identify lines having distinct characters. This will not only help in identifying the distinct lines but also demarcate the collection areas where additional exploration can be made.

The grass is largely apomictic with sparse nature of facultative apomicts. Despite of this, a high level of polymorphism has been reported (Chandra *et al.*,

2004). An estimate of existing variation within and between populations of species is useful for analyzing the genetic structure of crop germplasm (Hayward and Breese, 1993). The characterization of germplasm is required to maintain identity and purity for proper conservation and management. Further, this helps to identify unique lines of crop species, especially those growing unabatedly under harsh environmental conditions. Morphological, phenological and agronomic characteristics are often used for estimating genetic variations. These traits however, are often polygenic and/or influenced by environmental conditions.

Despite high variability at morphological level, variations in isozyme alleles in *Dichanthium* germplasm of the drier regions has not been studied. The allozyme technique (Hunter and Markert, 1957) provides an opportunity to establish genetic relationships among crop species and cultivars, and used to estimate genetic diversity in many crops (Tanksley and Orton, 1983; Kongkiatngam *et al.*, 1995; Cipriani *et al.*, 1996; Freitas *et al.*, 2000; Bhandari *et al.*, 2006; Brahmi *et al.*, 2004; Jain *et al.*, 2006). The considerable agro-morphological variation observed among accessions collected from similar eco-geographical situations (Agarwal *et al.*, 1999).

Traditionally, the evaluation of the genetic diversity or composition of germplasm have been conducted on the basis of morphological and phenotypic characters which are time consuming and blurred by environmental influences. Hence, these frequently lack the resolving power needed to identify individual genotypes. In the recent years DNA markers have been used extensively in genetic studies of many plant species. Development of various type polymerase chain reaction (PCR)- based marker systems and the invention of automated through-put genotyping instruments have created the opportunity to use molecular markers as practical tools for plant breeding. Many important traits of forage and turf grasses are controlled by quantitative trait loci (QTL). Improvement of quantitative trait through conventional breeding approaches is generally slow and difficult. The molecular marker technology makes it possible to construct high

density linkage map, thus making it possible to dissect quantitative traits into a set of discrete loci (Tanksley *et al.*, 1989). Marker analysis can measure the allelic effect on an individual locus of a quantitative trait. This attribute of molecular markers allows pyramiding the desirable alleles controlling a complex and/or intractable trait into in adapted genetic background. Estimation from biochemical markers, viz., isozyme analysis, may also be biased as only minor or coding portion of genome are taken into account hence less number of loci is represented by these markers. Isozyme provided the first molecular marker system for genetic study in plants. Isozymes are differently charged proteins molecules that can be separated by electrophoresis because they have same substrate specificity but different mobility (McMillin, 1993). Because enzymes catalyze specific biochemical reactions, the location of particular on the gel can be visualized by supplying the appropriate substrate and co-factors in a color producing reaction (Markert and Moller, 1959). Isozyme markers have been applied successfully in forage and turf grasses to distinguish between verities of *Lolium, Poa,* and *Trifolium* (Wehrer *et al.*, 1976; Weeden and Emmo, 1985; Lallemand *et al.*, 1991). Nevertheless, this has succeeded in developing the relationship amongst the germplasm to some extent and grouping them into broad categories, the potential for large scale use of isozyme markers for improvement of forage grasses is limited. In the last decade, DNA markers like restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and interspersed simple sequence repeats (ISSR), sequence tagged sites (STS) and random amplified polymorphic DNA (RAPD), have been used in genetic and breeding studies in many plant species (Williams *et al.*, 1993). Random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) have been used in cultivar analysis, species identification in most plants due to technical simplicity and speed of RAPD methodology (Gepts, 1993). Compared to restriction fragment length polymorphism (RFLP) markers, RAPD can generate markers more rapidly but some loss of information may occur because RAPD markers are usually dominant rather than co-dominant as RFLP markers. Our earlier studies in *Dichanthium* indicated a high level of variations with RAPD markers (Chandra *et*

al., 2004), molecular markers such as RFLP, RAPD, AFLP, etc. have been used to assess genetic diversity at the DNA level, allowing an estimation of the degree of relatedness between individuals without the influence of environmental variation. Among the many facets of DNA marker technology, DNA fingerprinting has been the most pervasive application. The attraction of DNA fingerprinting has been because of its increased power of resolution and the potential for absolute objectivity. Among the various techniques available, RAPD analysis is a potentially simple, rapid and reliable DNA fingerprinting method.

Apart from RAPD, inter-simple sequence repeats (ISSR) technology is also quite productive and easy, it is based on the amplification of region 100-3000bp between inversely oriented closely spaced micro satellites (Zietkiewicz *et al.*, 1994). The major advantage of this method is the fact that it does not require a time consuming steps of genomic (or other) library construction in spite of the fact that ISSRs are mostly inherited as dominant or rarely as co-dominant genetic marker. Transferability and application of STS markers also makes it suitable to finger print those crops where genomic or genomic based SSR are not available. In the present case many STS markers of *Stylosanthes*, which is a range legume, were attempted to finger print them for better characterization as the case with *Cenchrus* spp. (Chandra and Dubey, 2007).

Association of molecular marker with agronomic important traits can be achieved either by QTL analysis or using large numbers of accessions and perform the association study with DNA finger prints data. Pakniyat *et al.* (1997) demonstrated the association of salt tolerance characters viz., Na^+ and C_{13} with AFLP markers. Out of 204 polymorphic AFLP bands 12 were significantly associated with shoot sodium content and C_{13} where 6 out of 12 showed the presence of an AFLP band associated with low sodium content and more negative C_{13} and other six cases these effects were associated with the absence of an AFLP band. This strategy allows candidate genetic markers, genotypes and collection sites to be identified for a suitable trait (s) (Forster *et al.*, 2000). It has also been

reported that fruit weight is a key trait to success in tomato salt-tolerance improvement using QTL markers for total fruit weight under salinity of wild *Lycopersicon* germplasm (Monfort *et al.*, 1996). Since grasses are generally tough and faces extreme temperature and other environmental aberrations, it will be interesting to correlate some of the drought responsive characters namely osmolyte concentration, osmotic adjustment, injury index, proline level, total soluble protein, total sugar and specific leaf area etc. with the developed DNA patterns. The specific leaf area (SLA) has been found to be negatively associated with rate of transpiration (TE) which in turn associated with drought behavior in *Stylosanthes* (Thumma *et al.*, 2001).

Water is earth's most distinctive constituents and is essential ingredient of all life. Its deficit is most common environmental factor. Limiting crop productivity has devastating effect on crop yield. Certain stages such as germination, seedling and flowering, are most critical for water stress damage. Water stress reduces plant growth and manifest several morphological and biochemical alteration in plants ultimately leading massive loss in productivity.

Water stress inhibits protein synthesis, induce small sets of stress specific proteins, promote important modification in gene expression causes activation or inhibition of activities of many enzymes, and lead to change in the ultra structure of tissues. Various groups of investigators in the last few years reported the mode of protein synthesis in plant parts under water stressed environment (Zhang *et al.*, 1966; Close, 1997; Bewley and Larsen, 1982; Baker *et al.*, 1995; Dasgupta and Bewley, 1984) the level of proteins in stressed plants (Kumar and Singh, 1991; Rai *et al.*, 1994), and the activities of key enzymes influenced under water stress (Mali *et al.*, 1980).

Osmotic adjustment is generally regarded as an important adaptation to drought because it helps to maintain turgor and cell volume. Osmotic adjustment (OA) involves an active accumulation of solutes within the plants in response to

lowering of soil water potential, reducing the harmful effect of water stress (Morgan, 1984). An effective OA mechanism is now receiving recognition as a adaptive mechanism for stress. OA in higher plants can occur either through accumulation of solute or the breakdown of osmotically inactive compounds, one such solute is proline whose level under stress condition increases many folds in many plants. The solute that accumulates during osmotic adjustment includes sugar, amino acids, organic acids, proline and glycine betain (Hanson and Hitz, 1982; Jones and Turner, 1980; Munns and Weir, 1981). Osmotic adjustment has been shown to occur in a variety of monocots and dicots. One of the best characterized osmoregulatory responses in plants under stress is the accumulation of proline. In some tissues proline increases as much as 100 fold in response to stress (Voetberg and Sharp, 1991). The accumulation of proline results from an increased flux of glutamate to pyrroline-5-carboxylate by induction of pyrroline-5-carboxylate synthase (P5CS) in proline biosynthetic pathways as well as decreased rate of proline catabolism (Stewart *et al.*, 1977; Rhodes *et al.*, 1986). Elevated level of proline is expected to protect plant tissue against stress by acting as N-storage compounds, osmosolute, and hydrophobic protectant for enzymes and cellular structure (Le-Rudulier *et al.*, 1984). Water stress in plants often leads to oxidative damage in plants by inducing the production of active oxygen species and decreasing the activity of antioxidant enzymes like catalase, peroxidase and superoxide dismutase (Mali *et al.*, 1980).

Dichanthium spp. is one of the important constituents of Indian grasslands. The available diversity and their genetic potential for resistance to stress especially to moisture have not been properly assessed and recorded. Further, no attempt has been made using molecular markers to characterize similarities and dissimilarities in various accessions in this grass and also their association with drought responsive characters. Such studies are likely to make a dent in any future forage improvement programme.

Objectives of the proposed research work:

The grasslands available in India are mostly dominated by *Dichanthium* and *Dichanthium-Bortihocloa* complex species. To develop the pasturelands, there is always a need of having superior species and accessions of the grasses. To accomplish this, there is need to evaluate the available germplasm so that it can be used in improvement of grasslands. The present knowledge indicated that the available germplasm of *Dichanthium* have not been properly assessed at the biochemical and molecular levels. As such thin knowledge is available about the biochemical characteristics of *Dichanthium* grasses in limited water conditions and also their association with DNA markers. In the proposed study the developed DNA finger prints of *Dichanthium* will be correlated with appropriate and identified drought responsive characters. Therefore, to achieve the mentioned targets following objectives have been envisaged:

1.To determine the morphological and biochemical variations amongst germplasm and their behavior under limited water conditions.

2.To develop DNA fingerprints of *Dichanthium* germplasm.

3.To estimate and establish association between drought responsive characters and polymorphic DNA bands.

Review of Literature

Application of isozyme markers in genetic diversity studies:

Genetic relationship studies are better option of relating the biochemical characteristic among species, variations detected by molecular makers offers a number of advantages over morphological and biochemical as they are generally affected by environment and less representation of loci respectively. However, use of isozymes for cultivar identification is well documented and widely used for genetic relationship study. Ramirez *et al.* (1985) studied three isozyme systems (cathodal peroxidase, alkaline phosphates and alpha-amylase to characterize and classify six rye (*Secale cereale* L.) seed samples, study indicated that different electrophoretic patterns of isozymes allowed to distinguish each sample.

In contrast to leaf proteins, seed protein composition is generally not influenced by environment factors (Gardiner *et al.*, 1986) are used as valuable tool for cultivars identification. Wouters and Booy (2000) reported the identification of 68 cultivars of perennial ryegrass (*Lolium perenne* L.) by using and combining the results of esterase isozyme and total seed protein, the discrimination was based on quantitative differences (relative band intensity rather than qualitative differences), it was further reported that esterase pattern from different seeds of rye grass was very stable.

Fernandez *et al.* (2005) distinguished 29 cultivars of lily (*Lilium* spp.) by using eight isozyme systems. Some isozyme banding patterns (IBP) were identified as section specific biochemical markers. The cluster analysis indicated that the lily cultivars could be separated from other *Lilium* species, except for two *L. formonlogi* cultivars: 'Hakuba' and 'Hakuko' which could not be distinguished from each other by the isozyme banding patterns, hence isozyme can provide useful biochemical marker for cultivar identification and to estimate phylogenic relationship among those lily cultivars.

Messina *et al.* (1991) reported the utility of isozyme as genetic markers in cultivars identification of Kiwifruit (*Actinida deliciosa*). Fifty four entries putatively belonging to seven female and two male kiwifruit cultivars were examined for 13 isozyme system (AAT, ACO, GDH, G6PDH, 1DH, MDH, ME, MNR, NDH, 6PGD, PG1, PGM and SKDH). Four isozymes showed identical banding patterns (ACO, MDH, NDH and SKDH) and remaining enzyme systems showed best discriminating power. All the New Zealand cultivars were uniquely identified by simultaneous comparison of the AAT, PGI and PGM zymograms.

The isozyme marker system could be useful in genetic improvement and breeding programme involving inter specific crosses, Carrera and Poverene (1995) discriminated *Helianthus petiotaris* (A wild species) from *Helianthus annuus* by using 7 isozymes (ADH, ACP, EST, GDH, LAP, PGI and PGD).The pattern obtained were compared with zymograms of inbred lines, hybrids and open pollinated varieties of *H. annuus*. The same alleles for EST and SKDH isozymes were found in 60th species, while ACP showed an allele that has not been found is sunflower, the rest of the isozyme system showed both common alleles and characteristic ones for each species. ACP, GDH and PGD were monomorphic in *H. petiolarls*, while ADH and LAP were monomorphic in *H. annuus*. Verron *et al.* (1993) estimated the genetic distance and genetic diversity among five improved verities and one wild accession of lily of the valley (*Convallaria majalis* L.) by using isozyme marker system. Five isozymes namely esterase, acid phosphatase, peroxidase, phosphoglucomutase and superoxide dismutase. Esterase system gave lowest polymorphisms, peroxidase and phosphoglucomutase systems gave highest polymorphism. The study indicated that certain genetic diversity exists among the types of lily of the valley and the isozyme variations may be related to genetic variation.

Isozyme system has been also reported a very attractive marker system for breeding purposes. Avila *et al.* (2003) studied eighteen isozyme systems for evaluation and characterization of 147 accessions of *Vicia faba* from different

origins, including four known botanical types. The most polymorphic isozyme systems were AAT, FK, PGD, PRX and SOD, which provided a discriminatory tool for evaluating and characterizing collections. The study also detected possible contamination of or some degree of heterozygosity within certain inbred lines. The study discussed the use of allozymic variation in germplasm identification and characterization, breeding programs, and other genetic studies on the species (*Vicia faba*). Although molecular markers are now predominantly used for genetic relationship and trait association studies, isozyme analysis continues to be used as a relative simple and inexpensive method for obtaining genetic information for screening of large germplasm collection.

Application of DNA based molecular markers system in genetic diversity studies:

RAPD marker system:

Two decades ago, Bostein *et al.* (1980) described the first DNA profiling technique, RFLP and RAPD. The major areas of potential marker utilization were defined as:

(1) Varietals and percentage identification, (2) Identification of genetic loci affecting quantitative economic traits; and (3) Genetic improvement programme, including screening and evaluation of germplasm source resource, introgression, improvement of commercial hybrids and within population selection (Sollar and Beckmann (1983). The advent of PCR (Saiki *et al.*, 1988) and the resulting exponential increase of marker systems suitable for genetic analysis have given a substantial impetus both for the proliferation of genetic diversity studies, and the initiation of marker assisted selection.

The choice of marker system (s) for genetic diversity studies is driven by several considerations. These include the availability of markers, marginal assay cost, size of experiment and preference between a high average expected heterozygosity and a high effective multiplex ratio (Powell *et al.*, 1996).

DNA markers like restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) have been used in genetic and breeding studies in many plant species (Williams *et al.*, 1993). Random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) have been used in cultivar analysis, species identification in most plants due to technical simplicity and speed of RAPD methodology. Gepts (1993) compared to restriction fragment length polymorphism (RFLP) markers with RAPD, and elucidated that RAPD can generate markers more rapidly but some loss of information may occur because RAPD markers are usually dominant rather than co-dominant as RFLP markers. Our earlier studies in *Dichanthium* indicated a high level of variations with RAPD markers (Chandra *et al.*, 2004).

Ever since molecular makers become available in appreciable numbers, many studies have been directed at *Hordeum spontaneum,* the immediate wild ancestor of cultivated barley. Zhang *et al.* (1993) typed 268 accessions using isozyme and RFLP marker. While both RFLP and isozymes were highly polymorphic both within and among population, neither the number of alleles per locus nor the average level of diversity different with either technique. However, the relative amount of within Vs between population components varied greatly between the two marker systems. Isozymes revealed a large amount of within population diversity, where as RFLP resolved a higher proportion of between population and detected more heterozygosity. More recently Pakniyat *et al.* (1997) explored the potential of AFLP markers to estimate genetic diversity, as well as to genetically analyze various complex traits. Thirty nine genotypes of *H. spontaneum* belonging to three geographically separated areas of the fertile crescents discriminated on the basis of AFLP patterns. Owuor *et al.* (1999) studied on *H. spontaneum,* used RAPDs to demonstrate strong association between specific loci and soil types, between gene diversity and soil type, and in the frequency with which rare alleles were observed in one soil type over another. Molecular marker have not only proved efficient in the analysis of genetic diversity in space and across eco-geographic gradients but have also been

successfully used to test that common assertion that scientific plant breeding has led to a narrowing is crop diversity overtime (Reeves *et al.*, 1999; Donini *et al.*, 2000).

Punitha and Ranveendran (2004) assessed the genetic diversity in colored and selected white linted cottons using RAPD molecular marker system. A collection of 11 colored cotton (*Gossypium hirsutum*) genotypes and four white linted genotypes of different origin were evaluated using 32 different RAPD primers, high level of polymorphism (76.31) was detected the cluster analysis showed clear cut separation of colored and white linted genotypes and thus formed three clusters.

Nair *et al.* (2002) studied the genetic relationship and diversity in 28 prominent Indian sugarcane varieties cultivated under wide range of agro climatic conditions using 25 RAPD markers. The varieties belonging to the one parentage were grouped under different cluster white varieties from different percentages were grouped under the same cluster. The tropical and subtropical identifies of verities also did not contributed to the clustering pattern as individual clusters included varieties from both tropics and subtropics. This showed that genetically similar varieties are present in both regions.

Singh *et al.* (2006) used RAPD marker system for assessing genetic diversity and species relationship among 28 accessions of egg plant representing five species, these accessions were collected from different parts of country. In total 144 polymorphic bands were obtained from 14 random primers, the value of Jaccard's coefficient ranged from 0.05 to 0.82 dendrogram (by UPGMA) showed that *S. incanum* is closest to *S. melongena* followed by *S. nigrum* and genetically distinct genotypes were identified.

Mantzavinou *et al.* (2005) estimated genetic diversity of 19 Greek landraces and 9 cultivars of durum wheat [*Triticum turgidum* L.var. durum

(Desf.)] along with two commercial bread wheat cultivars and one genotype of *Triticum monococcum* L. by using RAPD method. In total 87 seven random primers were screened of that 15 were used for complete analysis. Out of 150 bands obtained 125 bands were polymorphic (82.3%). The cluster analysis revealed that all the genotypes were grouped in one broad cluster where as the *Triticum monococcum* L. cultivar stood apart from all other genotypes.

Molecular markers have clearly played an important role in diversity analysis (Karp *et al.*, 1997). Challenges for the future include the practical exploitation of such markers to relevant ecological and biological questions and for the establishment of core collection in germplasm banks (Gepts, 1995). Opportunities also exists to shift emphasis away from anonymous marker to genes of known function and to focus on factors determining adoptive variation. The narrow genetic base of crop plants is well documented. Tanksley and McCouch (1997) have highlighted the prospects for "unlocking genetic potential from the wild". This strategy is built around the principle that phenotype is not always a good indicator of genotype and that exploitation of genetic resource might be better based on the use of molecular markers. The proposal is that exotic material should be selected by identifying genotypes with the greatest number of unique alleles displayed by DNA profiling and the approach is of particular significance for character that show quantitative inheritance, since the genetic control of these can be complex. The application of molecular and genetic mapping will facilitate access to a broader spectrum of genetic variation and should play a significant role in promoting the sustainable use of genetic resources. Perhaps more importantly exploitation of naturally occurring variation will have an important role in unraveling gene functions (functional genomics). The major advantages of DNA marker over conventional marker system in that, they are practically unlimited in numbers, they are neither growth stage specific nor they are subjected to either environment interactions or to epistatic or pleiotropic effects.

ISSR molecular marker system:

Zietkiewicz *et al.* (1994) reported this technique for first time, where microsatellites anchored at the 3' end are used for amplifying genomic DNA. They are mostly dominant markers. Numbers of primers can be synthesized for various combinations of di-, tri-, tetra- and penta- nucleotides [e.g. 3^3=27, 4^4=256] with a few based anchors.

Hanna *et al.* (2005) well documented the efficiency of ISSR, SSR and SAMPL marker system in detecting genetic polymorphism among 30 winter rye inbred lines and compared the results of cluster analysis performed on data from these marker systems using different statistical methods and coefficients, it was further reported that each marker system was able to discriminated among materials analyzed with lowest value of average genetic similarity (GS) obtained with ISSR markers (0.288) and highest with SAMPLs (0.538). EST-derived SSR turned out to be less efficient in detecting genetic diversity. The average GS value for combined SSR data was 0.3569. Since correlation between similarity and Cophenetic materials was not obtained with various method systems, suggested that different marker system should be used for genetic diversity study to exploit as many sources of polymorphism as possible.

Sarla *et al.* (2003) reported the discrimination of geographically diversed *Orysa nivara* accessions and established genetic relationship using ISSR and SSR markers based on AG and GA repeats. Genetic diversity among 24 accession of *O. nivara* from 11 states of India and fair *O. sativa* verities one each from Glaszmanris isozyme group I, II, V and VI were analyzed using ISSR and SSR – PCR. The primers based on AG and GA repeats were informative; their resolving power ranged from 4.2 to 10.8 and PIC from 0.64 to 0.89. They could together enable grouping of accessions on a geographical basis. Ten alleles out of 40 amplified at 6 loci were unique to an accession. Two accessions each from UP and Bihar and one from M.P. were distinct from other accessions. *O. nivara*

alleles are common with Jaya, Pular, Basmati 370 and Taipei -309 were identified.

Sowframanien *et al.* (2002) studied the genetic variation in 12 gamma ray induced mutants in black gram by using RAPD and ISSR marker. In total 35 RAPD and 8 ISSR primers were used and polymorphism was detected. The percentage of polymorphism ranged from 12.5 to 50 for RAPD and 12.5 to 44.4 for ISSR. A significant DNA polymorphism among the mutants were observed using RAPD (28.8) and ISSR (33.3%) markers. A young leaf *Chlorina* mutant and a smooth pod mutant showed more DNA polymorphism as compared to the parent.

Among the various molecular markers, ISSR profiling is one of the most reliable tools extensively used is many crops plants to work out genetic relationship like Citrus (Fang and Roose, 1997). Vijayan and Chatterjee (2003) selected 11 mulberry (*Morus* spp.) cultivars, collected from wide range of agro-climatic conditions. ISSR marker system was selected for establishment of genetic relationship and association of marker with leaf yield. The genetic distance among the cultivars varied from a minimum of 0.053, between Punjab local and Bombay local to a maximum of 0.431 between Almora local and Sujanpur-5, three main clusters were obtained. The north Indian cultivars made a separate and distinct group while cultivars originated from eastern and southern India occupied a distinct position. Further, two markers (825.14 and 835.75) associated with leaf yield were also identified.

The microsatellite sequence based marker systems i.e., ISSR and SSR are known to be attractive tool for number of approaches including genetic diversity analysis due to their multiallelic nature, high reproducibility and locus specificity. previous sequence information is required for the development of SSR primers, which is a major draw back to their application in species, hence studied less intensively. ISSR technique in which polymorphism results from length

differences between inversely oriented closely spaced micro satellites is a co-effective alternative of SSR.

For many forage crops, characterization of varieties is often difficult due to a lack of reliable morphological traits and a high degree of intra-varietals variations. Isozyme markers have limited utility. Moreover molecular markers (RFLP, RAPD and AFLP) for forage in turf species do not seem convenient for routine description of verities. It is the same for microsatellites, which are likely the most interesting markers, but their development is expensive and at present few sequences are publicity accessible (Kubik *et al.*, 2001; Jones *et al.*, 2001).

STS marker system:

The good level of polymorphism of STS marker was shown by many studies. The studies of Bert *et al.* (1999) and Jones *et al.* (2002) have shown that the STS markers can be useful in genetic map construction. However, fodder grasses have been less intensively studied than other members of poaceae and few DNA sequences are developed in the data base.

Patricia and Lallemand (2003) developed 28 STS markers from *lolium* sequences especially using consensus sequences from related species of *Gramineae.* Primer pairs were designed in order to amplify the intronic regions thus, the polymorphism detected was based on intronic length polymorphism out of 42 STS markers development 85.8% yielded successful amplification and 62% revealed a high level of polymorphism. The analysis of amplicons revealed a high STS marker specificity, more over the majority of the STS markers can be considered as "universal markers" because 81% of these STS markers amplified successfully across 20 related grass species.

Sethy *et al.* (2003) reported the isolation of microsatellite sequences and their conversion to sequence tagged microsatellite sites (STMS) markers in chickpea (*Cicer arietinum* L.). 10 STMS primer pairs were utilized to analyze the

genetic polymorphisms in 15 *C. arietinum* verities and two wild verities, all primer pair amplified polymorphic loci ranging from four to seven alleles per locus with observed heterozygosity ranged from 0 to 0.6667. Most of the STMS markers also amplified corresponding loci in the wild relative, hence these markers will be useful for the evaluation of genetic diversity and molecular mapping in chickpea.

Beata *et al.* (2003) used 28 STS primers to screen of polymorphisms between two *L. sativus* accessions, ATC 80878 and ATC 80407 resistant and susceptible respectively to *Mycosphaerella pinodes* infection. Ten primer pairs revealed polymorphism between ATC 80878 and ATC 80407 when PCR product were digested with range of endonucleases, results suggested that the STS-based PCR analysis will be useful for generating useful molecular marker in *L. sativus*.

Application of molecular markers in trait association studies:

Mishra and Mandi (2004) reported the genetic diversity among 29 Darjeeling-grown tea clones using AFLP marker system. AFLP diversity estimates based on Jaccard's coefficient allowed separation of the 29 clones into three clusters. Genetic relatedness between the clones was found to be at 70% levels, further 11 selected clones were used to develop DNA finger prints using 11 random primer which generated 131 polymorphic bands, the activity of drought specific superoxide dismutase (SOD) and ascorbate peroxidase (APX) isozymes was found to be appreciably high in RR17/144, CPI, TV 26 and AV2. The association of specific isozyme activity peak areas a dependent variables and RAPD bands scored was independent variables, stepwise regression showed that the RAPD band (1400bp) obtained with OPAHO2 primer has a highly significant regression coefficient for Cu-Zn SOD activity (b = 0.970) and APX 11 activity (b = 0.968) using fisher's exact test (F-test). The association between Cu-Zn SOD and APXII and RAPD band of 1400bp was found to be significant at 99.9% confidence. Being associated with clones exhibiting high activity for drought tolerance

specific isozymes, this DNA band (marker) could be used in germplasm screening for drought tolerance in tea plants.

Nguyen *et al.* (2004) investigated the influence of genetic back ground on salt tolerance in *Acacia* species and also associated RAPD marker with salt tolerance. The seedlings of three provenances form each of *Acacia auriculiformis* and *Acacia mangium* were subjected to salt stress for one month. Plant growth, leaf osmotic adjustment and RAPD analysis were studied. In comparison to control plants, the plant growth in all provenances was decreased by salt stress and provenances A3 and M1 were more tolerant than others in *A. auriculiformis* and *A. mangium* respectively. Salt stress decreased leaf osmotic potential in all provenances. The difference in osmotic adjustment between the provenances was not correlated with the concentrations of minerals examined such as Na^+, K^+, and Ca^{2+} in both species, but it was correlated with the leaf proline concentration in *A. auriculiformis*. These results suggested that the provenance variation for salt tolerance can be partially accounted for by plant physiological measures. The genetic polymorphism between the provenances was detected by RAPD analysis. Thirty-nine out of 71 bands and 18 out of 63 bands detected were polymorphic for the provenances of *A. auriculiformis* and *A. mangium*, respectively. The similarity indices between the studied provenances were less than 65% in *A. auriculiformis* and 86% in *A. mangium* suggesting that the provenance variations for salt tolerance were mainly due to the difference in genetic background. The RAPD specific markers for each provenance were determined. These markers ~~can be~~ are considered as RAPD markers associated with salt tolerance in the two *Acacia* species.

Pakniyat *et al.* (1997) demonstrated the association of salt tolerance characters viz., Na^+ and carbon-13 with AFLP markers. Out of 204 polymorphic AFLP bands 12 were significantly associated with shoot sodium content and carbon -13 where 6 out of 12 showed the presence of an AFLP band associated with low sodium content and more negative carbon -13 and other six cases these

effects were associated with the absence of an AFLP band. This strategy allows candidate genetic markers, genotypes and collection sites to be identified for a suitable trait (s). Forster *et al.* (2000) reported that fruit weight is a key trait to successes in tomato salt-tolerance improvement using QTL markers for total fruit weight under salinity of wild *Lycopersicon* germplasm (Monfort *et al.*, 1996). QTL markers related to the drought resistance which are found on chromosomes of maize (Agrama *et al.*,1996). Since grasses are generally tough and faces extreme temperature and other environmental aberrations, it will be interesting to correlate some of the drought responsive characters namely osmolyte concentration, proline level, total soluble protein, total sugar and specific leaf area with the developed DNA patterns. The specific leaf area (SLA) has been found to be negatively associated with rate of transpiration (TE) which in turn associated with drought behavior in stylosanthes (Reddy *et al.*, 2000).

Zhou *et al.* (2006) identified a total of 12 drought related quantitative trait loci (QTL) by investigating drought tolerance of introgression lines (wild rice) under 30% PEG treatment at the young seedlings stage. Of these QTLs, the alleles of 4 QTLs on chromosome 2, 6 and 12 from *Dongxiang* common wild rice were responsible for increased drought tolerance of the introgression lines. In particular, a QTL qSDT12-2, near RM17 on chromosome 12, was consistently detected in different replications, and expressed stably under PEG stress throughout the study. It was also found that the QTLs located on different chromosomes might express at different stages.

Physiological / biochemical aspects of the plants under water stress:

Osmotic adjustment:

Cellular responses to water deficit include loss of ~~larger,~~ turgor change is plasma membrane fluidity and composition, changes in water activity and/or solute

concentration and protein-protein-lipid interaction (Bray, 1997; Heide and Poolman, 2000). Several metabolites that play important role in stabilizing enzyme complex, protecting membranes, and ensuring the osmotic adjustment (OA) required to maintain turgor, are synthesized in response to drought. Osmotic adjustment has been considered as one of the crucial process in plant adaptation to drought stress, because it sustains tissue metabolic activity and enables re-growth upon rehydration but varies greatly among genotypes e.g. it is more important in rice or wheat than in maize (Morgan, 1984). However in term of crop yield there are not many field studies showing a consistent benefit from osmotic adjustment (Quarrie *et al.*, 1999). Osmotic adjustment as normally a slow process and is triggered above a certain threshold of cell water deficit. The osmotic compounds synthesized includes proteins and amino acids (like proline, aspartic acid and glutamic acid) (Samuel *et al.*, 2000; Hamilton and Heckathorn, 2001), methylated quaternary ammonium compounds (e.g., glycine betaine and alanine-betaine) (Rathinasabapathi *et al.*, 2001; Sakamoto and Murata, 2002), hydorphilic proteins (e.g. late embryogenesis abundant, LEA), carbohydrates like Fructans and Sucrose (Vijn and Smeekens, 1999) and Cyclintons (e.g. D-pinitol, mannitol) (Anderson and Kohorn, 2001).

The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines was well documented by Patakas *et al.* (2002), where water relations, gas exchange as well as organic solute and ion accumulation were studied in the leaves of 2-year-old grapevines (*Vitis vinifera* L, cv. Savatiano) grown under well watered (control) and water stress conditions. Both osmotic potential at full turgor (Π_{100}) and at turgor loss point (Π_0) decreased significantly in stressed plants compared with the control. Photosynthetic rate, and stomatal conductance were also significantly lower in stressed plants. Starch concentration decreased almost threefold in stressed plants, while there were no significant differences in sugar accumulation between the two treatments. Total inorganic ion concentration increased rapidly in stressed plants and seems to be the major component of osmotic adjustment in stressed grapevines. Thus, the energetic

cost of osmotic adjustment in grapevines using inorganic ions would be expected to be much lower than for those species using organic solutes (Patakas *et al.*, 2002).

Accumulation of solutes under stress not only decrease cell osmotic potential, but also allowing maintenance of water absorption and cell membrane and metabolic machinery under dehydration. Compatible solutes exert their protective activity by interacting with water molecules, favoring the reduction of solvent protein-membrane interaction can lead to the stabilization of protein complex and membranes (Bohnert *et al.*, 1995).

Claudio *et al.* (2006) reported the divergent selection for osmotic adjustment that resulted in improved drought tolerance in maize (*Zea mays* L.) in both early growth and flowering phases, the maize (*Zea mays* L.) cultivars of similar genetic background were taken and results obtained using crops of two S4 populations derived from a cross between inbred lines exhibiting the highest and lowest capacities for osmotic adjustment in a screening applied to 20 inbred lines. The mean values of OA for the two S4 populations were 0.47 MPa for the high OA population (HOA) and 0.06 MPa for the low (LOA). Crops of these populations were grown under a rain-out shelter and subjected to 30-day droughts either before or during flowering. In both experiments, exposure to drought evoked a significant ($p < 0.05$) decrease in osmotic potential measured at full turgor in the HOA population, no change was found in the LOA population. This induced response became evident in plants of the HOA population in measurements effected 16–18 days after suspension of irrigation. Irrespective of the timing of drought, the HOA crops extracted significantly more water from deeper in the soil profile during the stress period, exhibited higher leaf area duration and attained greater grain yields and, in the crop subjected to water stress at flowering, greater harvest index than the LOA crops. The components of yield and their determinants (i.e., floret number per ear, grain set, grain number and weight per grain) exhibited differential responses with timing of the drought and

in response to level of OA. Under irrigation, there were no differences between populations in either experiment in terms of yield and its components, or in harvest index, leaf area duration, or soil water extraction. This conclude that OA can contribute to drought tolerance in maize crops exposed to water deficit both before and during flowering, and that the trait carries no yield penalty under irrigation.

It should be pointed out that osmoprotection mechanism are not probably functional until severe dehydration occurs with the implication that osmotic adjustment may be critical to survival rather than to increase plant growth and crop yield under drought stress. However, certain reports favor that OA can be related with yield parameters.

Moinuddin *et al.* (2004) reported the role of osmotic adjustment in drought tolerance of Chickpea (*Cicer arietinum* L.) and its relation with seed yield and yield parameters, eight cultivars of differing in OA capacity were field grown. The cultivars were divided into high and low OA groups and crop performance was assessed group wise at three growth stages, as a result, water potential, osmotic potential and RWC decreased progressively with increasing soil moisture stress and age of the crop. As compared to low OA cultivars, high OA cultivars proved significantly superior to low OA ones in seed yield and most of its parameters. The yield and most of its parameters, the yield benefit was 26 and 48% at moderate and sever moisture stress level. This coincided with osmotic adjustment ranging from 0.28 to 0.48 MPa and from 0.37 to 0.71 MPa, respectively at various phases of reproductive additionally, a positive relationship between OA and grain yield in water deficit environment has been shown in grain Sorghum (Ludlow *et al.*, 1990), (Basnayake *et al.*, 1995) in wheat (*Triticum aestivum* L.) (Morgan *et al.*, 1986; Blum *et al.*, 1999), barley (*Hordeum Vulgare* L.) (Blum, 1989), chickpea (Morgan *et al.*, 1991) and pigeonpea (*Cajanus cajan* L.) (Subbarao *et al.*, 2000a).

Many reports regard OA to be causal mechanism favoring crop productivity under water deficit environment. However, there are also some conflicting reports indicating a negative relationship between OA and seed yield under drought (Grumet *et al.*, 1987; Subbarao *et al.*, 2000b). Other reports indicate no relationship between OA and growth and/or seed yield in field conditions under drought (Snackel and Hall, 1983; Munn, 1988; Ludlow, 1986).

Subbarao *et al.* (2000a) studied the pattern of osmotic adjustment in Pigeonpea (*Cajanus cajan* L.) for drought tolerance and its effect on yield under drought. Twenty six extra-short-duration pigeonpea [*Cajanus cajan* (L.) Millsp.] genotypes were grown with irrigation during the growth period or with water deficit imposed from flowering until maturity. Significant genotypic variation was observed in the initiation of OA, the duration of OA and the degree of OA. Based on the measured OA at 72, 82, and 92 days after sowing (DAS), genotypes were grouped into five different clusters. Genotypic differences in total dry matter production under drought were positively associated with OA at 72 DAS (r^2=0.36**, n=26). Significant positive relationship between OA at 72 DAS and grain yield under drought was found (r^2=0.16*; n=26). However, OA towards the end of pod filling phase, i.e. at 92 DAS, had a significant negative relationship with grain yield under drought (r^2=0.21*; n=26). Genotypic differences in grain yield under drought was best explained using stepwise multiple regression to account for differences in OA at 72, 82, and 92 DAS (r^2=0.41**; n=78). The degree of OA at 72 and 82 DAS contributed positively to the grain yield, whereas OA at 92 DAS contributed negatively to this relationship. Since OA is considered as adoptive Mechanism for of plant for drought stress. Need to determine where OA can be used as selection criterion for screening drought resistant cultivars. Jongdee *et al.* (2002) demonstrated the relationship between OA and low water potential (LWP) in rice, when data were combined across experiments for vegetative and flowering stages. Under water-limited conditions around flowering, grain yield reduction was mainly due to a increased spikelet sterility. Variation in OA was neither related to grain yield nor yield components. There

were however, negative phenotypic and genetic correlations between LWP and percentage spikelet sterility measured at flowering stage on panicles at the same development stage during a water deficit treatment. This suggests that traits contributing to the maintenance of high LWP minimized the effects of water deficit on spikelet sterility and consequently grain yield.

Protein level under water stress:

The levels as well as soluble proteins are altered in plants growing under water deficit environment compared with plants growing under non stressed conditions. Various workers have observed either a decrease (Kumar and Singh, 1991; Hsiao, 1973; Barnett and Naylor, 1966; Yu *et al.,* 1996) or increase (Kumar and Singh, 1991; Rai *et al.,* 1983) in the level of total or soluble proteins in different organs of plants subjected to water stress. The increase or decrease level of protein depends on the plant species and organ studied as well as the severity of the stress. Shah and Loomis (1987) observed decrease content of soluble and total proteins in sugar beet leaves. When Bermuda grass plants were subjected to increasing water stress a decrease in soluble protein level was observed (Barnett and Naylor, 1966). The decrease level of the total soluble protein content in water stressed plants (Kumar and Singh, 1991) appears to be due to more degradation of proteins as well as overall inhibition in protein synthesis under water stress. Genotypes of crop cultivars differing in water stress tolerance, show different levels of total soluble proteins as well as specific activity of protease. Seedlings of drought tolerant moung bean genotypes showed higher protein content as compared with drought sensitive genotypes at -10 barr moisture stress (Kumar and Singh, 1991). Similarly, drought resistant maize (*Zea mays* L.) cultivars show a high protease activity at higher level of water stress, where as inhibition in protease activity in was noticed in sensitive cultivar at same magnitude of water stress (Thakur and Thakur, 1987). While comparing the total protein and free amino acid pool size in drought resistant and drought sensitive cultivars of *C. arietinum* and *Z.mays*, (Rai *et al.,*1983) observe the resistant plants are characterized by an increase over non stressed plants in total protein and free

amino acid levels. A genotypes of *C. arietinum* cv C-214 showed an increase of 60% protein over control at an osmotic potential of -3atm, whereas a sensitive cultivar, G-130, showed a 15% increase over control under similar conditions of water stress. Similarly, drought resistant *Z. mays* cv Ageti-76 plants noticed 190% increase in protein content over control, these observations indicated that that water stress has varying effect on the level of protein in different species, and the stress- induced response depends on the species of crop examined, and it may vary even in different organs within the same species.

Proline responses:

One of the most studied compatible solute is amino acid proline. In plants, proline is synthesized in the Cytosol and mitochondria from glutamate via Δ'Pyrroline 5-carboxylate (P5C) by two successive reduction catalyzed by P5C Synthetase (P5CS) and P5C reductase (P5CR) respectively (Hare *et al.*, 1999).

The accumulation of various solutes in the cell to decreases the osmotic pressure of cell for turgor maintenance is known as osmo-regulation sugars and amino acids are major constituents in plants of these, usually there is a remarkable increase in free proline amino acid. Khan *et al.* (1983) studied among free amino acids, proline contributes about 30% fraction of the total amino acid pool. Badapati *et al.* (1992) reported that proline content increased bye 10 to 100 times higher in stressed plants than the non stressed plants. Moisture stress caused by drought is invariable attended by free proline accumulation in different plant types from bacteria or algae to higher plants. Proline accumulation under stress seems to be depended on light intensity and reserve carbohydrate in plants. Arora and Saradhi (1995) reported the light induced enhancement in proline level in *Vigna radiata* when exposed to environmental stress. Further it was also suggested that photosynthetic activity of seedling might be responsible for the light induced enhancement of proline level. Joyce *et al.* (1992) reported that light enhances the proline accumulation in water stressed leaves of barley and the response was found to be linked with photosynthesis. Talwar *et al.* (2002)

observed and reported the effect of high temperature on proline accumulation, in different parts and processes of plants i.e., floral parts, invitro pollen germination, pollen vigour and role of proline in protecting pollen from heat stress in three groundnut (*Arachis hypogea* L. genotypes, it was concluded that heat injury during floral development of sensitive genotype may be due to decline in proline content during early floral development stage and inhibition in the transportation of proline from author wall to pollen. Tholkappian *et al.* (2001) studied the impact of water deficit and corresponding change in proline content in mycorrhizal and non mycorrhizal soyabean, where lower level of proline was recorded in mycorrihizal soyabean than non mycorrihizal at moisture stress. The nitrogen content and pod number per plant increased in mycorriihizal soyabean than non-mycorrhizal plants at 25 percent moisture levels, though accumulation of proline under stress have been demonstrated in helping plants against desiccation, increase in proline level have negative effect, suppressing the rubsico activity in higher plants (Siva kumar *et al.,* 1998).

Francisco (1998) studied the response of 49 Pea cultivars with different drought tolerance. The tolerance to stress was determined according to the grain yield or the harvest index in rainfed farming. In these conditions variability among the genotypes in turgor maintenance, measured as the slope of the turgor potential (ψ_p) function against water potential (ψ_w). The cultivars, which best maintained turgor, were those which were more drought-tolerant. Turgor maintenance was significantly related to osmotic adjustment (OA). The free proline level increased (from 4 to 40 times) in response to water stress. However, the contribution of this amino acid to ψ_{s100} was small (approximately 1%) and no significant relationship was observed between proline content and OA. The cultivars which accumulated more proline had lower water contents upon turgor loss. This seems to indicate that proline may play a role in minimizing the damage caused by dehydration.

Abdel Hamid *et al.* (2003) studied the well documented effect of exogenous application proline on the expression of ubiquitin, anti oxidative

enzymes and dehydrins that improve the adaptation of *Pancratium maritium* L to salt stress. The result indicated that the salt stress brought about a reduction of the growth and protein content, particularly at 300mM NaCl. It was significantly increased by exogenous proline, and severely salt stress resulted in an inhibition of the antioxidant enzymes catalase and peroxidase activity, but the activity of these enzymes was maintained significantly in presence of proline.

The role of glycine betaine and proline in improving plant abiotic stress resistance was reviewed by Ashraf and Foolad (2007). Aida *et al.* (2005) studied the over expression of Δ'-Pyrroline-5-Carboxylate synthase increases proline production and confers salt tolerance in transgenic potato.

Proline accumulation appears to be mediated by both ABA- dependent and ABA independent signaling pathways although the events that occur between the perception of stress and induction of proline biosynthetic genes are poorly characterized. Recent evidence supports an important role for post transcriptional event in dehydration and ABA-induced proline synthesizes (Hare *et al.*, 1999).

Accumulation of soluble sugars, free amino acids and proline during stress plays important role in osmotic adjustment in Sorghum (Yadav *et al.*, 2002). Increase in the level of solutes and decrease in leaf water potential, solute potential, relative water content (RWC), stomatal conductance and chlorophyll content under stress recovered to normal level after 24 hrs of re-watering the plants.

Claussen (2005) studied the reliability of proline level in measurement of stress in tomato. Tomato (*Lycopersicon esculentum* Mill. cv. Counter) plants were grown in nutrient solutions containing equal nutrient ratios at increasing concentrations (X1 = standard concentration, and X3, X5.5, X8 or X11 = 3, 5.5, 8 of 11 times the standard concentration). Fruit yield decreased significantly from moderate (X3) to high (X11) nutrient concentration during summer, but remained nearly unaffected by the strength of the nutrient solution under low-radiation conditions in autumn. This stress-induced difference in yield was reflected by

higher proline concentrations in leaves of plants grown during the summer compared to those grown during the late season. The decrease in fruit fresh and dry weight observed in summer was due to reduced availability of water and the distribution of dry matter towards the vegetative plant parts at the expense of reproductive growth. The proline content of tomato leaves fluctuated according to nutrient concentration and total radiation, and was closely related to the relative water content of leaves. It was concluded that proline is a reliable indicator of the environmental stress imposed on plants, thus allowing us to establish stress thresholds for fruit yield and product quality of hydroponically grown tomato.

Transgenic tobacco containing osmotin gene induces over expression of proline under both drought and salinity stresses, relative water content and photosynthesis were also seen high in transgenic plant than that of wild type (Gupta *et al.*, 2001). Water stress was found to reduce diurnal leaf water potential and leaf osmotic potential in 60 genotypes but leaf osmotic potential was significantly higher in drought tolerant cultivars C-306 than in the drought sensitive cultivars Kalyansona. Carcellor *et al.* (1999) studied the water stress around anthesis on proline accumulation and translocation from leaves of two maize cultivars DA 4F37 and DA XL 636) and reported that water stress increased leaf proline content only is DA4F37, while proline in leaf exudates was detected only DA XL636 water stressed plants. High proline concentration during morning was found in leaves with high relative water content this report also support that proline is involved in osmotic adjustment.

Sunita *et al.* (1991) reported proline status of genetically stable salt tolerant *Brassica juncea L.* somaclones and their parent cv. Prakash. The *Brassica juncea* L. somaclone (SR- 1, 2, 3) selected in vitro for NaCl-tolerance, Non selected somaclones (CP-5) and parent cv. Prakash, and were characterized for their free proline content in absence of stress and as a function at increasing salt stress.

Phutela *et al.* (2000) studied the water relations, proline content and activities of Pyrroline -5- Carboxylate Synthetase (P5CS) and proline oxidase in five *Brassica juncea* genotypes under drought tolerance, it was found that drought tolerant cv Varuna showed maximum osmotic adjustment 1/b of 3.21 and proline content increased from 8.4 and 5.3 μ mol/g dry weight under normal conditions to 128.2 and 44.5 μ mol/g dry weight under stress conditions in the leaves and roots respectively. The increase in proline content in less drought tolerant variety Prakash was from 7.0 and 1.8 μ molμ mol/g dry weight in the presence of stress in roots and leaves respectively.

Enzyme activities:

Normal metabolism of plant under water stress condition is adversely affected and con-current disturbance of the enzymatic constitution of the plants. Water stress lowers the level of many enzymes in the tissue. (Mali *et al.*, 1980; Geigenberger *et al.*, 1997) water stress leads to oxidative damage in plants by inducing the production of active oxygen species and decreasing the activities of the antioxidant enzyme like catalase, peroxidase and superoxide dismutase (Mali *et al.*, 1980). The Magnitude of reactive oxygen species (ROS) production and anti oxidant activity decided the tolerance/susceptibility of genotypes (Sairam *et al.*, 2003). The over production of antioxidant enzymes provides an elegance approach to engineer plant species genetically for water stress tolerance. The transformation of alfalfa plants expressing Mn-superoxide dismutase cDNA from *Nicotiana* spp. have been shown to be more resistant to drought stress (McKersie *et al.*, 1996).

The oxidative burst during which large quantities of reactive oxygen species (ROS) like super oxide, hydrogen peroxide, hydroxyl radicals, peroxy radicals, alkoxy radicals, singlet oxygen etc. are generated in one of the earliest responses of plant cell under various abiotic and biotic stresses and natural course of senescence. The imposition of both abiotic and biotic stress causes over production of ROS, which ultimately impose a secondary oxidative stress in plant

cell. Degradation of membrane lipids, resulting in accumulation of free fatty acids, initiates oxidative deterioration by providing a substrate for enzyme lipoxygenase, causing membrane lipid peroxidation. Since lipid peroxidation is known to produce alkoxy, peroxy radicals as well as singlet oxygen, these reactions in the membrane are major source of ROS in plant cell. ROS-induced oxidative damage and their protection by anti oxidative system is discovered, ROS like H_2O_2 acts as a signaling molecule, second messenger, mediated the acquisition of tolerance to both biotic and abiotic stresses (Souman Bhattacharjee, 1995).

Shao *et al.* (2005) selected 10 kinds of wheat genotypes and reported the anti-oxidative results of maturation stage in terms of activities of POD,SOD CAT and MDA content as follows: (1) 10 wheat genotypes were grouped into three kinds (A–C, respectively) according to their changing trend of the measured indices; (2) A group performed better resistance drought under the condition of treatment level 1 (appropriate level), whose activities of anti-oxidative enzymes (POD,SOD, CAT) were higher and MDA lower; (3) B group exhibited stronger anti-drought under treatment level 2 (light-stress level), whose activities of anti-oxidative enzymes were higher and MDA lower; (4) C group expressed anti-drought to some extent under treatment level 3 (serious-stress level), whose activities of anti-oxidative enzymes were stronger, MDA lower; (5) these results demonstrated that different wheat genotypes have different physiological mechanisms to adapt themselves to changing drought, whose molecular basis is discrete gene expression profiling. POD, SOD, and CAT activities and MDA content of different wheat genotypes had quite different changing trend at different stages and under different soil water stress conditions, which was linked with their origin of cultivation and individual soil water threshold.

Ismail *et al.* (2005) investigated the changes in plant growth, relative water content (RWC), stomatal conductance, lipid peroxidation, proline and antioxidant system in relation to the tolerance to polyethylene glycol mediated water stress in drought sensitive common bean *P. vulgaris* L. accession FM 53

and drought tolerant tepary bean *P. acutifolius* gray accession PI 321–638. For induction of water stress, the 35 days old bean seedlings were subjected to PEG 6000 of osmotic potential −0.40 MPa for 14 days. With regard to vegetative growth, PEG treatment caused more decrease in *P. vulgaris* than in *P. acutifolius* indicating a superior performance of wild species under water stress. Root and shoot DW increased in *P. acutifolius* while decreased in *P. vulgaris* on day 14. PEG treatment had no effects on relative water content (RWC) in *P. acutifolius* but reduced RWC in *P. vulgaris*. *P. acutifolius* maintained a greater stomatal conductance than *P. vulgaris* under water stress imposed by PEG treatment. In *P. acutifolius* constitutive level of lipid peroxidation was lower than in *P. vulgaris* and did not change at the end of the experiment. Constitutive activities of SOD, CAT, APOX and POX were higher in *P. acutifolius* than in the sensitive one and SOD, APOX and GR activities showed an enhancement in the former under water stress. Proline accumulation was also higher in *P. acutifolius* than in *P. vulgaris* both under control and water stress conditions. These results possibly suggest that the drought tolerant tepary bean *P. acutifolius* showed a better protection mechanism against oxidative damage by maintaining higher constitutive and induced activities of antioxidant enzymes, than the sensitive common bean *P. vulgaris*.

A numbers of antioxidant enzymes such as superoxide dismutase, peroxidase and catalase. The superoxide anion, which is the most dangerous reactive oxygen species, is scavenged in plants by superoxide dismutase (SOD EC 1.15.1.1) which converts superoxide anion to hydrogen per oxide .Hydrogen peroxide (H_2O_2) is scavenged directly by catalase (CAT EC 1:11:1:11) which convert it to water and molecular oxygen peroxides such as ascorbate peroxidase (APX, EC 1.11:1.7) also scavenge H_2O_2 indirectly by combining it with antioxident compounds such as ascorbate and guaiacol. Measurement of activities of antioxident enzymes can be used to indicate oxidative stress in plants.

Isozyme in stress:

Several isozymes like superoxide dismutase, catalase, GR, peroxidase and APX are present in plants and their relative composition changes during exposure to stress (Pinhero *et al.,* 1997). The intensity of many SOD isoforms increased after imposing different stress and it is reported that intensity of SOD-4 isoform was greater than rest of the isoforms after 20 hour of chilling stress in maize (Pinhero *et al.,* 1997) it was reported that esterase and acid peroxidase produced specific and new isozyme bands and the lack of the several isozymes in drought resistant somaclones as compared with the parental cultivars of wheat. Guan and Scandalios (1998) reported that although both sod-4 and SOD-4A transcript accumulates during late embryogenesis, only sod-4 is up regulated by ABA and osmotic stress, it was observed that accumulation of SOD -4 transcript in response to osmotic stress in a consequence of increased endogenous ABA levels in developing embryos. It was also hypothesized that the increase the SOD mRNA in response to ABA is due to in part to ABA metabolic changes leading to changes in oxygen free radicals level, which in turn lead to the induction of the antioxidant defense system. Dubey (1983) reported decrease number of amylase isozyme due to salinity in rice. Mishra *et al.* (1993) reported that the disappearance of peroxidase isoforms in response to salinity and it was specific to the organ (root and shoot) of tolerant and susceptible lines of green gram. Peroxidase isoforms has been used as sensitive marker for saline stress. Sheoran and Garg, 1997; Chang, 1984) Polymorphism of peroxidase and esterase have been detected in seedlings of the salt sensitive and resistant sunflower varieties, increased activity of peroxidase was also observed in the seedlings of the resistant varieties while it is reduced in the sensitive varieties.

Materials and Methods

Plant Materials:

Thirty *Dichanthium* accessions used for the present study represented collections from north central plateau (Bundelkhand region of Lalitpur and Chatterpur district of UP and MP) and southern plateau and hills (Dharwad and Bangalore, Karnataka) (Table 1). These accessions were collected from their natural habitats *i.e.*, grasslands and open fields and maintained in experimental fields of the Indian Grassland and Fodder Research Institute, Jhansi. The fresh and young plants from well grown and maintained tussock were transferred and grown in pots of uniform size (Figure 1). The tussocks were kept in uniform size by regular trimming. Fresh and young leaves from well grown tussocks of thirty *Dichanthium* genotypes in triplicate were studied to investigate their performance for various physiological and biochemical attributes under control and water stress conditions imposed by withholding the water. Molecular study was performed by isolating DNA from individual plants of each accession.

Morphological Observations:

Morphological data were recorded from well grown tussocks at 50% flowering stage.

Following observations were recorded and they were categorized as qualitative and quantitative traits.

Quantitative trait:

1. **Tussock height:** It was measured from base to top of the plant and recorded.
2. **Number of tillers per tussock:** Total number of tillers was counted and recorded.

Table 1: Agro-climatic regions and place of collection of 30 accessions of *Dichanthium annulatum* used in the present study.

No.	Accessions no.	Agro-climatic regions	Associated characteristics of regions
1	IG97-234	North India central plateau	Arid to semi – arid, soils are alfisols and antisols, annual rainfall 800-1000 mm but restricted to the months of July, August and September and regions are characterized by very hot dry summers, maximum temperature touches 48 °C
2	IG97-24	Lalitpur, north India	
3	IG95-30	Lalitpur, north India	
4	IG97-192	North India central plateau	
5	IG97-247	North India central plateau	
6	IG97-241	North India central plateau	
7	IG95-25	Lalitpur, north India	
8	IG95-114	Lalitpur, north India	
9	IG97-147	North India central plateau	
10	IG97-170	North India central plateau	
11	IG97-151	North India central plateau	
12	IG97-152	North India central plateau	
13	IG97-184	North India central plateau	
14	IG97-158	North India central plateau	
15	IG97-118	North India central plateau	
16	IG97-233	North India central plateau	
17	IG97-189	North India central plateau	
18	IG97-132	North India central plateau	
19	IG97-218	North India central plateau	
20	IG97-130	North India central plateau	
21	IG97-121	North India central plateau	
22	IG97-244	Chatterpur, north India	
23	IG97-245	Chatterpur, north India	
24	IG97-144	Chatterpur, north India	
25	IGKMFD-1	Dharwad, south India,	Humid, mixed red and gravelly soils, annual rainfall 700 – 1000 mm but distributed over a wide time period
26	IGTGD-4	Dharwad, south India	
27	IGBANG-D-2	Bangalore, south India	
28	IGKMD-10	Dharwad, south India	
29	IG3108	Dharwad, south India	
30	IGBANG-D-1	Bangalore, south India	

Fig. 1. *Dichanthium* accessions maintained in pots under net house conditions.

3. **Internodal length:** Length between two nodes were measured in centimeter.

4. **Longest leaf length and width:** It was measured in centimeters at the widest portion of leaf blade of the third leaf from the top of the main tiller. Average leaf width in millimeters from three leaves was calculated accordingly.

Qualitative traits:

- **Inter nodal color:** Color of nodes was categorized in three types as Red, light yellow and light green
- **Nodal hairs:** Hairs on nodes were recorded as less, Medium and dense.
- **Awns:** Present or absent, if present then it was further divided into less, medium and dense.
- **Pits on floret**: Recorded as absent or present.
- **Culm thickness:** Culm thickness was measured by using vernier calipers in millimeter (mm).
- **Number of spikes and spikelets per plant at flowering stage:** Total number of spikes present in one plant was recorded.
- **Rachis hairiness:** Rachis was observed for presence or absence of hairs.
- **Type of stem:** It was determined by cutting stem transversely with sharp scalpel and recorded as solid or hollow.
- **Leaf hairiness:** Leaf surface was observed and recorded as either hairy or smooth.
- **Habitat:** Recorded as erect, semi erect and creeping.

Measurement of leaf water relation parameters:

Quantification of crop water status was made by measuring the leaf water relation parameters i.e., water potential (WP), osmotic potential (OP) and relative water content (RWC) at well irrigated condition (control) and under water stress (withholding of water for period of time).

Relative water content (RWC):

Relative water content (RWC) was determined by following the method of Barrs and Weatherley (1962). The expended and fresh leaves were cut into a uniform discs and weighed (Fresh weight). The leaves discs were than floated on distilled water for 6-8 hours at room temperature under the intense luminous light and determined the turgid weight by properly soaking leaves discs by tissue paper and weighed (Turgid weight), the same discs were oven dried at 80°C for 48 hour and dry weight was measured. RWC was calculated using the following equation.

RWC = (FW-DW) / (TW-DW) x 100

Where FW is fresh weight, TW is turgid weight and DW is dry weight

Measurement of leaf water potential (WP):

Leaf water potential was measured at mid-photoperiod (10-12 noon) in fully expended leaves which were cut into uniform discs in three replicates from three plants of single genotype with thermocouple psychrometer and Wescor C-30 chambers connected to HR33T Dew point microvolt meter (Wescor Inc. USA) Water potential was recorded which is linear function of electro magnetic force produced by the temperature difference between the junction at the dew point temperature and the ambient temperature. The recorded value was later divided by proportionality constant (-0 75μ moles /bar) to get the value of water potential in bar which were further converted into Mega Pascal (MPa).

Measurement of osmolality and osmotic potential:

Osmolality was measured using leaf samples. Fresh and young leaves were collected and freezed in sealed pack polythin bags at -20°C. After 2 days the leaves were thawed at room temperature. The cell sap was extracted using compressed chamber (model LP-27, Wescor, USA) directly on the filter paper discs, and osmolality of cell sap was measured with a vapour pressure osmometer (5500,Wescor, Inc USA). Osmometer was calibrated with known concentrations (mmoles/kg) of NaCl solutions. Values obtained indicated the osmolality (mmoles/ kg).

These values were further converted into pressure units (MPa) using the following equation.

OP (MPa) = -R x T x moles /kg (osmolality)

Where R is gas constant (0.008314) and T is the temperature (Kalvin Scale)

The obtained OP was further corrected for the dilution of symplastic sap by apoplastic water by using equation (OP+0.1 OP), Assuming 10% apoplastic water (Kramer, 1983). The osmotic potential obtained at full turgor denoted as OP_{100} was calculated according to Wilson *et al.* (1979) using the following equation.

OP_{100} = (corrected OP x RWC)/100

Measurement of osmotic adjustment (OA):

Osmotic adjustment was calculated as the difference in osmotic potential at full turgor (OP_{100}) between stress and control treatments in each genotype.

Estimation of proline:

Estimation of low molecular weight free proline amino acid was determined in 500mg fully expended leaves in 3% sulphosalicylic acid in both control and stress plants by the method given by Bates *et al.* (1973). Five hundred milligrams leaves sample were homogenized in 10 ml of 3% aqueous sulphosalicylic acid. The homogenates was filtered through Whatman No. 2 filter paper. Two ml of filtrate was taken in a test tube and 2.0 ml of glacial acetic acid and 2 ml of acid – ninhydrin (1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation until it is completely dissolved, stored at 4^0C and used within 24 hrs.) were added and mixed. The mixture was heated at 100 ^{0}C for 1 hour in boiling water bath. After incubation reaction was terminated by placing the tube in ice bath. Four milliliter toluene was added to the reaction mixture and stirred well for 20-30 sec followed by extracting the solution using the separating funnel. Toluene containing proline was collected. Optical density (OD) was measured at 520 nm wave length. Standard plot of proline was made using pure proline solution of different concentration in place of tissues extract following the same procedure.

Estimation of malondialdehyde (MDA):

It was estimated in leaf tissues by following the procedure of Heath and Packer, 1968. Two hundred fifty milligram fresh leaf samples were homogenized in 5 ml cooled distilled water to fine slurry and homogenate was collected in 15 ml centrifuge tube. In it 5 ml TBA-TCA reagent (containing 0.5% thiobarbituric acid and 20% trichloroacetic acid) was added to the homogenate and mixed gently. After mixing the slurry was incubated in water bath at 95^{0}C for 30 min. The reaction was terminated by placing the tubes in ice bath for 10 minutes, and finally centrifuged at 10,000 rpm for 10 min. The supernatant was collected carefully and OD was recorded at 600 nm and 535 nm. Lipid peroxidation was determined by MDA content produced by thiobarbutric acid (TBA) reaction at low pH. The pink chromogen was measured at 535 and 600 nm for correction of the blank. The unspecific turbidity was corrected by substracting A_{600} from A_{535}. Lipid peroxidation was expressed as milimoles per liter.

Estimation of total soluble proteins:

Proteins in ground leaf samples for enzymes, isozymes are estimated following the Lowry method (Lowry *et al.*, 1951). In one ml of water 5 µl enzyme extract was taken and in the same test tube 5 ml of reagent C was added to each tube. After 10 min. of incubation 0.5 ml of Folin-coicalteau reagent was added and mixed well. All tubes were incubated at room temperature in dark for 30 min. In blank instead of enzyme extract water was taken. Blue color developed and OD was recorded at 660 nm. Standard graph of protein was constructed using known amount of bovine serum albumin (BSA) to calculate the amount of protein in unknown samples.

Cell membrane stability (Injury index):

Twenty five uniform leaf discs were taken in Pyrex tube containing 10 ml deionized distilled water and incubated at 25°C for 24 hr, then the electrical conductivity of the leachate was measured at 25°C directly using reading conductivity meter in both stressed and control (non stressed) tissues.

The tissue with leachate was then autoclaved, cooled to room temperature and total electrolyte was again measured by conductivity meter.

The percentage membrane injury (I %) was calculated according to the formula of Blum and Ebercon (1981).

(% Membrane injury) I =1-[(1-T1/T2) / (1-C1/C2)] x 100

Where C1 and C2 represents readings of electrical conductivity of control samples before and after autoclaving them respectively, and T1 and T2 represent readings of electrical conductivity of water stressed samples before and after autoclaving them respectively.

Determination of Enzyme activity:

Preparation of enzyme extract:

Fresh and young leaves were collected in icebox (4°C) for enzyme extraction. Plant samples were homogenized in three fold volume of cold extraction buffer (consisted of of 50 mM Tris-HCl, pH 7.2, 10 % sucrose, 1.0 mM EDTA, 20mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol (added freshly) and a pinch of polyvinyl pyrrolidone (PVP) in pre chilled pestle mortar to very fine slurry.. The homogenate was centrifuged at 12,000 rpm for 20 min. The supernatant obtained was referred as enzyme extracts and used as enzyme source. It was kept in ice till the assay was carried out. An aliquot of the extract was used for protein determination (Lowry *et al.*, 1951) and used to determine the specific activity of the enzymes by dividing the total obtained units in one gram fresh weight by total milligram protein in one gram fresh weight.

Superoxide Dismutase (SOD, E.C. 1.15.1.1)

The SOD activity was determined by measuring its ability to inhibit photochemical reduction of Nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977) with suitable modifications. The 3.0 ml reaction

mixture contained 0.05M Na_2CO_3, 0.1mM EDTA, 63µM NBT, 13.0µM methionine, 20µl enzyme extract and 1.3µM riboflavin, riboflavin was added in last. The test tubes were placed under intense fluorescent light at sufficient distance at room temperature. After 20 minutes lights were switched off and OD was measured at 560 nm wavelength. The non irradiated samples were served as control. The reaction mixture lacking enzyme will develop maximum color due to maximum reduction of NBT. The reduction of NBT was inversely proportional to the enzyme activity. Thus to obtained ΔA, A_{560} of particular set was deducted from A_{560} of blank set (without enzyme). Percentage activity reduced was calculated and converted into units/min presuming.

50% inhibition = 1.0 unit

Peroxidase (POD, E.C.1.11.1.17).

Peroxidase activity was measured by the method of Chance and Machly (1955) where guaiacol was used as the substrate.

Guaiacol + H_2O_2 ⟶ Oxidized guaiacol + $2H_2O$

The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product was measured as peroxidase activity (POD) activity. In a 5 ml cuvette, 3 ml (0.1M) potassium phosphate buffer (pH-7.2) containing, 0.1ml (20 mM) guaiacol solution, 0.03 ml (12.3 mM) of hydrogen peroxide was taken. In it 0.05 ml of enzyme extract was added mixed and immediately placed ~~the cuvette~~ in sample chamber of the UV-VIS spectrophotometer against the reference which contains all the constituents except the enzyme extract. Increase in absorbance with time was recorded at A_{436}. The OD was measured at 15 seconds intervals for three min. This was done till the straight line appeared. Activity was determined as change in 0.01 OD per minute (one unit). Further it was divided by proteins content in mg present in extract to get specific activity ~~unit per mg protein~~.

Pyrroline 5-carboxylate synthetase (P5CS):

Pyrroline 5-carboxylate synthetase (P5CS) activity was measured by following the method of Hayzer and Leisinger (1980). 0.1 ml supernatant was taken in a test tube followed by addition of 250 µl of assay mixture (containing 50 mM L-glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM hydroxylamine, 50 mM Tris buffer pH 7.0). The tube was incubated at 37 ^{0}C for 30 min. After incubation the reaction was terminated by adding 2.5% (w/v) $FeCl_3$ and 6% TCA in (2.5 M HCL). The protein precipitated was removed by centrifugation and absorbance of the supernatant was measured at 535 nm. The amount of γ-glutamyl hydroxamate produced was measured from the molar extinction coefficient of 250 l mol^{-1} cm^{-1} from the Fe^{3+}- hydroxamate complex. All tubes were taken in triplicate and absorbance values were corrected for the values obtained at zero incubation time. The enzyme activity is represented in micromole min^{-1} mg^{-1} protein.

Polyacrylamide gel electrophoresis (PAGE):

Native polyacrylamide gel electrophoresis:

Discontinuous polyacrylamide gel electrophoresis was performed using a system based on Laemmli (1970).

Assembling the apparatus and preparation of gel solution:

Thoroughly cleared and dry glass plates along with proper spacers were fixed into gel casting unit (Banglore Genei, India) and tightly fixed with screw. The bottom of gel plates were sealed with 0.8% solution of molten agarose by gently pouring from sides of spacer with glass pipette and allowed the agar to solidify, resulting the sealing of bottom of glass plates. Two kinds of slab gels were casted based on their applications. They were either 1.5 mm or 1.00 mm thick and about 13 cm long and 14 cm wide when casted. Taking the account of size of the glass plates and thickness of spacer the amount (volume) of gel required was calculated. A 40 ml of resolving (separating) gel was prepared by mixing the various constituents. The required quantity of gel components were used for different percentage of gels preparation as given in table below.

Composition of resolving gels:

Constituents	7%Gel	10%Gel	12% Gel	16%Gel
1.5M Tris-HCl buffer (pH 8.9)	10.0 ml	8.0 ml	10.0 ml	10.0 ml
Acrylamide solution (30%)	9.2 ml	13.3ml	16.0 ml	21.3 ml
TEMED	20µl	20µl	20µl	20µl
Ammonium per sulphate (APS) 10%	200µl	100µl	100µl	100µl
*SDS (10%)	400.0µl	400.0µl	400.0µl	400.0µl
Final volume	**40.0 ml**	**40.0ml**	**40.0ml**	**40.00 ml**

After mixing all the constituents, gel solution was carefully poured between the two glass plates, with leaving sufficient space for stacking gel. After pouring the gel it was over layered with fine layer of butanol. Gel was left for 30-45 min at room temperature for polymerization. After polymerization, over layered butanol was poured off and top of gel was washed with 3-4 times with deionized distilled water and drained completely all the fluids from top of the gel, remaining water was soaked with blotting paper. Stacking gel was prepared by mixing gel components along with desired concentration of acrylamide as given in table.

Composition of 4% and 5% stacking gel:

		4%	5%
Water (distilled and deionized)	-	6.01 ml	6.9 ml
1.014M Tris-Cl buffer (pH 6.8)	-	2.5 ml	1.3 ml
Acrylamide solution (30%)	-	1.33 ml	1.7 ml
Ammonium per sulphate (APS) 10%	-	50.0µl	50.0µl
TEMED	-	10.0µl	10.0µl
*SDS (10%)	-	100.0µl	100.0µl
Final volume	-	10.0ml	10.0ml

* Used when SDS gel electrophoresis was performed otherwise maintained the volume with distilled water.

Since the polymerization begins as soon as the TEMED added, immediately gel mixture was mixed gently and poured stacking gel solution directly on to the polymerized resolving gel, immediately after pouring cleaned teflon comb of defined well numbers and thickness was inserted into stacking gel solution making sure that no air bubble was trapped. Gel was kept in vertical position for 20 to 25 minutes at room temperature for polymerization. Comb was removed carefully form polymerized gel and wells were washed with distilled water and sealing agarose was removed. Gel plate was now installed in electrophoresis apparatus. Filled the anodal and cathodal reservoirs of apparatus with Tris-glycine electrode buffer. If any bubble that trapped at the bottom of the gel between glass plates was removed carefully.

Electrophoresis of gels:

Gels were electrophoresed at low temperature (4°C) to resolve isozyme. About 150 µg of protein sample mixed with 5 µl of bromophenol blue (tracking dye) was loaded into the well from cathodal end. The gel was run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was ½-1 cm away from bottom.

Reagents:

1. Acrylamide and N, N'- methylene bis-acrylamide (30% stock solution):

Stock solution containing 29% (w/v) acrylamide and 1% (w/v) N, N'-Methylene bis-acrylamide prepared in deionized distilled H_2O and filtered before storing in dark brown bottle. Stock solution stored at 4°C.

For 100 ml volume:

Acrylamide	=	29.0 g
Bis-acrylamide	=	1.0 g

Dissolve in 60 ml H_2O and finally made volume up to 100 ml.

2. Tris-buffer stock:

After the Tris base has been dissolved in distilled H_2O adjust the pH of solution using HCl to 8.9 and 6.8 for resolving and stacking Tris- buffer respectively.

3. N,N,N,N, - Tetramethylethylene diamine (TEMED) :

Accelerates the polymerization of acrylamide and bis-acrylamide by catalyzing the free radicals from ammonium persulphate. Used directly as supplied.

4. Ammonium per sulphate (APS) stock solution (10%):

Stock solution was prepared by dissolving 1.0g Ammonium per sulphate in 10.0 ml distilled H_2O and stored at 4°C Fresh solution was prepared weekly.

5. Tris-Glycine electrophoresis buffer (5X): Stock solution (5X) was prepared by dissolving 3.0 g Tris base and 14.0 g of glycine in 900 ml distilled H_2O. In it 0.1 % SDS added freshly in case of SDS PAGE.

2.8 g Glycine and 0.6 g Tris base and (1.0 g SDS)* was dissolved in 990 ml distilled water and final volume was made up to 1000ml to get working solution of running buffer.

* In case of SDS PAGE.

6. SDS stock solution (10% (w/v): 100 ml.

10 g SDS was dissolved in 60 ml D. H_2O and final volume was made up to 100 ml with distilled H_2O and stored at room temperature.

7. Composition of 1X SDS gel loading buffer:

50 mM Tris-Cl pH 6.8

100 mM 2- Mercepto ethanol

2% SDS

0.1% Bromophenol blue.

10% Glycerol.

SDS -Polyacrylamide gel electrophoresis (PAGE):

SDS polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulphate (SDS) is an anionic detergent, which binds strongly to, and denatures proteins.

The number of SDS molecular bounds to a polypeptide chain is approximately half the number of amino acids residues in polypeptide chain. The protein-SDS complex carries net negative charge, hence move toward anode and the separation of protein is based on the size and molecular weight of protein.

The procedure of gel preparation and electrophoresis was same as discussed in native polyacrylamide gel electrophoresis, except that gel solutions i.e., resolving, stacking gel, sample loading butter and electrophoresis buffer contained 0.1% w/v SDS. Prior to loading the protein sample on the gel, protein samples were mixed with sample gel loading buffer to 1X, and boiled at 100°C for 3 min in boiling water bath followed by cooling the sample in ice. Immediately cooled samples were then loaded on to the wells.

Each well was loaded with approx 200 ng protein and gel was run initially for 10 min at 200 V and thereafter at 100 V until sample traveled through stacking gel and stack at the junction of blacking and resolving gel. Once stacking was completed gel was run at 200 V for 4-5 hrs. Till tracking dye reached to the bottom of gel once the run was completed gel plates were removed from electrophoresis apparatus and gels were separate from glass plated and kept for staining.

Staining of native protein gel:

Native protein gel was stained by immersing the gel in 100 ml staining solution containing methanol: distilled H_2O and acetic acid (4:5:1 ratio), 0.01% w/v brilliant blue R-250 (usually left for overnight). After staining gels were washed with distilled H_2O and immersed in destaining solution containing methanol: distilled H2O and acetic acid (4:5:1 v/v) ratio for 1-2 hrs with occasional shaking.

Staining of SDS-gel:

Prior to staining SDS gel, it was immersed in 100 ml fixative solution (10% Trichloro acetic acid) for 1-2 hrs, followed by washing the gel with 2 to 3 times with distilled H_2O and gel was immersed in staining solution as discussed in

native protein gel staining followed by de staining of in destaining solution. Destained gels were removed carefully and examined under light viewer.

Agarose gel electrophoresis:

Agarose is a linear polymer composed of alternating residues of D-and L-galactose joined by α-(1 → 3) and β (1 ⟶ 4) glycosidic linkages. The chain of agarose form the helical fibres that aggregate into super-coiled structures with a radius of 20-30nm. Gelation of agarose results in a three dimensional mesh and channels whose diameters range from 50 nm to >200 nm

Preparation of gel:

Gel casting tray was cleaned and dried, adages of casting tray was sealed with gel sealing tape and set the casting tray on horizontal bench. Followed by comb of desired size and wells number was fixed into casting tray in such as manner that the comb was slightly above the surface of tray and fixed with comb stand.

Gel solution of agarose was prepared in electrophoresis buffer (0.5 x TBE) at appropriate concentration (Table) in screw cap bottle. Agarose was mixed in 0.5X TBE buffer and slurry was heated in microwave oven slowly with occasional swirling till all the grain of agarose were dissolved and gave clear transparent solution, transfer the bottle into a water bath at 65°C. When molten gel was cooled to 65°C. Ethidium bromide was added to final cone of 0.5 μg/ml (10 mg/ml) and mixed with gentle swirling. Luke warm agarose gel solution was carefully poured onto the gel casting tray and allowed the gel to set at room temperature. Gel took 30-45 minutes at room temperature to set completely. Once gel was set completely, comb and sealing tape was removed carefully. Casting tray was mounted into electrophoresis tank and filled the tank with 0.5 x TBE buffer till gel completely submerged into the buffer. DNA sample was mixed with 6X bromophenol loading dye to a final concentration of 1X and sample was loaded into the slots (wells) of the submerged gel using micropipette. Closed the lid of tank and electrical cords were connected so that the DNA would migrate

toward the anode (Red lead). Gel was allowed to run at 80V till bromophenol blue dye traveled the desired distance. After run, the gel was placed on gel documentation (Alpha Innotech, USA) system under UV light to examine. Photographs of ethidium bromide stained gel were taken.

Protein extraction for enzyme activity assay:

Fresh young leave from well grown tussocks were collected from both control and stressed plants and brought them to laboratory in ice bucket. Five hundred milligram leaves sample was ground in pre chilled pestal mortar in 1.5 ml of pre-chilled Tris-HCl (50 mM Tris-HCl, pH 7.5, 10 % sucrose, 1.0 mM EDTA and 1 mM 2-mercaptoethanol (added freshly) extraction buffer in presence of 20 mM PMSF and pinch of PVP powder. The sample was homogenized to fine slurry and centrifuge at 12000 rpm for 20 min at 4°C. The supernatant was collected and required quantity was used for isozyme and rest of extract was stored at 4°C.

Polyacrylamide gel electrophoresis for isozyme:

The analysis of four isozymes viz., peroxidase (POD, E.C. 1.11.1.17), esterase (EST, E.C. 3.1.1.2), polyphenol oxidase (PPO, E.C. 1.14.18.1) and superoxide dismutase (SOD, E.C. 1.15.1.1) was carried out using native polyacrylamide gel (10 %) electrophoresis method (Laemmli, 1970) with discontinuous buffer system. Gel was run at low temperature to resolve isozyme. About 150 µg of protein sample mixed with 5 µl of bromophenol blue (tracking dye) was loaded into the well from cathodal end. The gel was run at 100 V till dye crossed the stacking gel and then at 200 V till the dye was ½-1 cm away from bottom.

Gel was detached from the glass plates and placed in distilled water to avoid exposure to air and a piece of gel was cut at the right hand corner to mark the side. Gel was incubated in staining solution (substrate), the zones where the enzymes located in the gel were visualized due to the appearance of colored reaction product. Gels were photographed by using Olympus OM 2000 camera.

Staining of gels:

Peroxidase (POD, E.C. 1.11.1.17):

The gel was stained as described by Veech (1969). Gel was incubated in a solution containing 100 ml 0.05 M sodium acetate buffer (pH 5.6) containing 100 mg benzidine (dissolved by boiling) at room temperature. In the same buffer 10.0 ml 3% hydrogen peroxide was added gently mixed and 5 ml acetic acid was added to develop the color. The bright blue colored bands appeared. When the band was stained sufficiently, the reaction was arrested by immersing the gel in the large volume of 0.67% sodium hydroxide and 7% acetic acid solution for 10 min.

Esterase (EST, E.C. 3.1.1.2):

The gel was stained as described by Wendel and Weeden (1989). The gel was washed with 0.05M sodium phosphate buffer two to three times at 5-10 min intervals. Finally gel was immersed in 100 ml 0.05M sodium phosphate buffer (pH 6.0), and incubated at room temperature for 30 min. In an eppendorf tube 20 mg of alpha naphthyl acetate was dissolved in 1 ml of 60% acetone and in the same tube 50 mg fast blue RR salt was dissolved and poured onto already incubated gel. The gel was now incubated at 37^0C for 20-30 minutes in dark with occasional shaking. Dark brown color bands appeared in the gel when desired intensity of band color was developed, the reaction was stopped by adding the mixture of methanol, acetic acid, water and ethyl alcohol in the ratio of 10:2:10:1 The stained gel was photographed using Olympus OM 2000 camera.

Polyphenol oxidase (PPO, E.C. 1.14.18.1):

The gels was stained as described by Wendel and Weeden (1989).When the bromophenol blue dye touched the bottom of the gel it was removed and equilibrated in 100 ml 0.1M potassium phosphate buffer (pH 7.0) containing 0.1% p- phenylenediamine for 30 minutes at room temperature. When the incubation

was over, pyrocatechol was added to final concentration of 10mM in the same buffer. Dark brown bands appeared was photographed.

Superoxide dismutase (SOD, E.C. 1.15.1.1):

The gel was stained as described by Wendel and Weeden (1989). After the run was over gel was removed from the gel assembly and incubated in dark for 30 minutes in 100 ml of 0.05 M Tris-Cl buffer (pH 8.0) containing 2 mg riboflavin, 1 mg EDTA and 10 mg NBT. After the incubation gel was shifted to bright and intense light for 30 minutes and then gel was washed with distilled water. The bands appeared in form of negative bands against the blue background was photographed.

Zymogram construction:

The zymogram of gels was prepared by measuring the distance of each band from the point of separating gel and relative mobility (R_m) of each band was calculated as the ratio of distance traveled by the band to the tracking dye. Bands were numbered on the basis of increasing R_m values. Loci and alleles were subsequently numbered and lettered respectively. The isozyme patterns were defined by taking into account the numbers and positions of bands.

Isolation of genomic DNA:

Genomic DNA was isolated following N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB) method (Iqbal *et al.*, 1997) with suitable modifications. Two grams of fresh and young leaves were ground in liquid nitrogen to fine powder and mixed with 4.0 ml CTAB total DNA extraction buffer (CTAB 2 % w/v, NaCl 1.4 %, Tris-HCl 100 mM pH 8.0, EDTA 20 mM and 2-Mercaptoethanol 100 mM, added freshly) and incubated at 65 ^{0}C for 1 hr with occasional swirling. Slurry containing nucleic acid was extracted with one volume of chloroform-isoamyl alcohol (24:1) then centrifuged at 8000 rpm for 15 min. Nucleic acid was precipitated by the addition of 0.6 volume of iso-propanol and kept at –20 °C for

2-3 hr. Nucleic acid pallet was dissolved in 10 mM TE buffer pH 8.0 and kept over night at 4°C. Dissolved nucleic acid was treated with 3ul/ml (5mg/ml stock) RNase by incubating them for 30 min at 37°C. After 30 min of incubation it was extracted twice with phenol/chloroform and finally with chloroform. DNA was precipitated using absolute ethanol and DNA pallet was finally washed with 70 % ethanol. Pallet was air dried and suspended in 1.0 ml TE buffer. Quantity and quality of DNA was quantified by UV spectrometry. OD was taken at A_{260} and A_{280} and A_{260} / A_{280} ratio was determined. Genomic DNA was visualized on 0.8 % agarose gels. Part of stock DNA was finally diluted with TE to final concentration of 5ng/µl for use in PCR.

Agarose gel electrophoresis and restriction of genomic DNA:

The DNA was electrophoressed on 0.8% agarose gel using 0.5X TBE buffer at 70 volts till loading dye was sufficiently traveled to visualize DNA in gel. After run the gel was stained in ethidium bromide. Along with the isolated DNA known amount of λ DNA digested with Hind III was loaded to check the size and amount of unknown DNA. The brief run was enough to reveal the quality and quantity of DNA. Five microgram (µg) of genomic DNA was restricted with Eco RI and Hind III in a final volume of 50 µl reaction mixture using 1X Eco RI and Hind III buffer respectively. The restriction of DNA was carried out at 37°C for 6-8 hrs. The restricted DNA was electrophoressed using 0.7% agarose gel containing ethidium bromide. The Hind III digest λ DNA was used as molecular weight marker. The gel was run at 70 volt using 0.5X TBE electrode buffer. When run was over the gel was photographed using Polaroid and SLR camera.

Polymerase chain reaction PCR:

Random Amplified polymorphic DNA (RAPD):

RAPD-PCR reactions was performed in 20 µl of reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM $(NH_4)_2SO_4$, 0.45 % v/v BSA, 3.5 mM $MgCl_2$, 150 µM of each dATP, dTTP, dCTP, dGTP, 7.5 pmoles (15ng) primer, 0.5 unit

Taq polymerase and 25 ng genomic template DNA. PCR product was visualized with ethidium bromide after electrophoresis on 1.6 % agarose gel.

Composition of reaction mixture for single RAPD Reaction (25µl):

10X PCR buffer(SM)	- 2.0µl	
MgCl 2 (25mM)	- 2.8µl	
dNTPs mix (10mM each)	- 1.25µl	
Primer (10mM)	- 1.2µl	
Taq DNA Pol(3U/µl)	- 0.15µl	(0.5unit)
H2O	- 10.5µl	
Genomic DNA	- 5.0 µl	(Diluted DNA to 5ng/µl to get 25ng)

Mixed all the components (except genomic DNA) and 15ul of the mixture was added in each single reaction.

The reaction was performed in thermal cycler (PTC 200, MJ Research, USA) with cycling program

Cycle- 1

94°C - 1.0 min

37°C - 1.0 min

72°C - 2.0 min

Repeat above steps in cycle-1 for 40 times

Cycle-41st

72 °C for 10 min (Extension)

and finally 4 °C for ever. Amplified products were stored at 4 °C.

Agarose gel electrophoresis for RAPD-PCR product: PCR product was separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul 5xDNA

loading dye and total samples were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 70V for four hours. The amplified product was visualized under UV trans-illuminator and was photographed using Polaroid and SLR camera.

STS PCR Procedure:

The PCR amplification of STS loci was conducted following the procedure given by Liu *et al.*, (1996) with certain essential modifications like adjustment of genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification in thermal cycler.

STS-PCR reaction was performed in 25ul volume reaction mixture containing 78.2 mM Tris-HCl pH (8.0), 19.4 mM $(NH_4)_2SO_4$, 233ug/ml BSA,0.53% TritonX-100 5.8 mM $MgCl_2$, 130 µM of each dATP, dTTP, dCTP, dGTP, primer, 1.0 unit Taq polymerase and 25 ng genomic template DNA.

Composition of reaction mixture for single STS-PCR:

10X PCR buffer$^{(SM)}$	-	3.3µl
$MgCl_2$ (25mM)	-	6.6µl
dNTPs mix (10mM each)	-	2.9µl
Primer (10mM)	-	2.0µl (1.0µl Reverse and Forward primer each.)
Taq DNA Pol(3U/µl)	-	0.3µl (1.0 unit)
H2O	-	10.5µl
Genomic DNA	-	5.0µl (Diluted DNA to 5ng/µl to get 25 ng)
Final volume	-	25µl

Mixed all the components (except genomic DNA) and 20ul of the mixture was added in each single reaction.

PCR conditions for STS-PCR. Following programme was used in PCR amplification for STS loci.

94°C - 60 second

55°C - 60 second

72°C - 90 second

Repeat above steps for 32 cycles

One cycle at 72°C for 5 min and finally at 4°C for ever. Amplified product was stored at 4°C.

Agarose gel electrophoresis for STS- PCR product:

PCR products were separated on a 1.6% agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul (5x) DNA loading dyer and were loaded on the gel. Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was ran at 80 volts for four hours. The amplified product were visualized under UV trans-illuminator and were photographed were taken using gel documentation system.

ISSR-PCR Procedure:

ISSR-PCR reaction was performed in 25ul volume reaction mixture containing 67.0 mM Tris-HCl pH (8.0), 16.6 mM $(NH_4)_2SO_4$, 4.0µg BSA,0.396% TritonX-100 2.0 mM $MgCl_2$, 150 µM of each dATP, dTTP, dCTP, dGTP,0.4µM primer, 1.0 unit Taq polymerase and 25 ng genomic template DNA.

Composition of reaction mixture for single ISSR-PCR

10X PCR buffer (SM) - 2.5 µl

$MgCl_2$ (25mM) - 2.0 µl

dNTPs mix (10mM) - 1.5 µl

Primer (10mM) - 2.0 µl

Taq DNA Pol(3U/µl) - 0.3 µl (1.0 unit)

H2O - 11.7 µl

Genomic DNA - 5.0 µl (Diluted tamplet DNA to 5ng/ul to get 25 ng)

Final volume - 25.0 µl

Mixed all the components (except genomic DNA) and 20ul of the mixture was added in each single reaction.

Following programme was used to amplify ISSR markers.

Cycle -1

94°C - 2.0 min.

Cycle- 2

94°C - 30 second

50°C - 1.0 min.

72°C - 1.30 min

Repeat above steps in cycle-2 for 34 times

Cycle-35

72 °C for 10 min

And finally at 4 °C for ever. Amplified product was stored at 4 °C

Agarose gel electrophoresis for ISSR-PCR product: PCR product was separated on a 1.6 % agarose with ethidium bromide in the gel using 0.5X Tris borate EDTA (TBE) buffer. Total reaction mixture was mixed with 2ul 5X DNA loading dye, and were loaded on the gel. Along with the unknown samples 20 base pair DNA ladder was also loaded to know the size of the amplified products. The gel was run at 80 volts for five hours. The amplified product were visualized under

UV trans-illuminator and were photographed were taken using gel documentation system.

Data analysis and development of dendrograms:

For isozyme markers the parameters estimated for genetic variation were percentage of polymorphic loci (Pp), mean number of alleles per polymorphic locus (Ap), mean expected heterozygosity based on unbiased estimate of Nei (1978) and allele frequency. Allele frequencies were used to estimate expected heterozygosity. A locus was considered polymorphic when more than one allele was found in an accession. The proportion of polymorphic loci was calculated by dividing the number of loci polymorphic by the total number of loci analyzed. The mean number of alleles per polymorphic locus was determined by summing all the alleles observed at polymorphic loci and dividing this sum by the number of polymorphic loci (Hamrick and Godt, 1997). Genetic diversity (H_e = Hardy-Weinberg expected heterozygosity) (Weir, 1989) was calculated for each locus (including monomorphic and polymorphic loci) by:

$$H_e = 1 - \Sigma x_i^2$$

Where x_i is the mean frequency of the i^{th} allele pooled across the accessions.

The binary data generated on the basis of presence (1) and absence (0) of the bands in isozyme, RAPD, STS, and ISSR, was analyzed for genetic similarity among the accessions based on Dice's similarity coefficients (s), which was also converted to distance measures (d) using the formula d = 1-s. Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using the Un-weighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. A RAPD band was considered polymorphic if the band was present in some accessions and absent in others, monomorphic if the band was present among all the accessions.

Polymorphic information content (PIC) value was calculated of individual primer to evaluate discriminatory power of markers. The PIC value was calculated applying the formula of Roldan-Ruiz *el al.* (2000): $PIC_i = 2f_i(1-f_i)$, where f_i is the frequency of the amplified allele (band present) and $(1-f_i)$ is the frequency of the null allele (band absent) of marker *i*. Marker index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.*, 1996).

Boot strap analysis:-

Boot strap analysis further authenticated the genetic relationship among the accessions. The reliability of dendrogram was evaluated with 1000 Boot straps using Win Boot software (Yap and Nelson, 1996).

Correlation of biochemical data with DNA patterns:

Significance of the value:

Standard excel software was used to calculate the degree of freedom, LSD, mean of square, t-test single and two way ANOVA.

Association of RAPD, ISSR and STS DNA finger prints with stress responsive traits.

Regression analysis was conducted using the data of stress responsive traits and data of three molecular marker systems viz., RAPD, STS and ISSR. In total eight stress parameters were used for association in all 30 *Dichanthium* accessions. These parameters were osmotic adjustment (OA), osmolality, MDA content, proline content, injury index, peroxidase activity, superoxide activity, and P5CS activity. Association of RAPD, STS and ISSR markers with these eight stress responsive traits were investigated using multiple regression analysis. The amount of change in the traits were treated as dependent variables and various RAPD, STS and ISSR bands (scores as 1 for presence of band and 0 for absence) were treated as independent variables. The regression analysis was based on the model:

$Y=a+b_1m_1+b_2m_2+....b_jm_j+...b_nm_n+d$,

Where Y is the trait (parameter), m the RAPD, STS and ISSR markers, b partial regression coefficient, d the between accession residue which is left after regression.

Solutions and buffers for plant DNA isolation:

1) 0.5M EDTA(pH 8.0): Dissolved 37.22g EDTA di-sodium salt and 4.0g Sodium hydroxide in 150 ml H_2O and adjusted the pH by NaOH solution, final volume was made up to 200 ml.

2) 1M Tris-Cl pH (8.0): Dissolved 24.23g Tris base in 150 ml distilled water Adjust to the pH 8.0 by HCl and made up final volume to 200 ml with water.

3) 5M NaCl: Dissolved 58.44g NaCl in distilled water and made up final volume to 200 ml with water.

4) CTAB (10%) W/V: Dissolved 200g CTAB in 180 ml distilled water. CTAB was dissolved by warming the solution. The final volume was made up to 200 ml.

5)10mM TE Buffer (pH 8.0): 2ml Tris-HCl from 1M Tris–HCl stock and 0.4 ml EDTA from 0.5M EDTA stock The final volume was made upto 200ml with D. water .

6) 5x Tris Borate EDTA Buffer or TBE Buffer (pH 8.0) : Dissolved 27g Tris base, 13.75g boric acid, 10ml (0.5M) EDTA in 400ml distilled water and made to final volume to 500 ml with water.

7) DNA Lysis Buffer(CTAB) :

Final concentration	Stock concentration
20mM EDTA	8ml (0.5M EDTA)
100mM Tris- HCL	20ml (1M Tris-Cl pH8.0)
1.4M NaCl	56ml (5M NaCl)
2% W/V CTAB	40ml (10% CTAB)
100mM 2-ME Added freshly	

Mixed and made up final volume to 200 ml with D. water.

8) DNA loading dye (Bromophenol blue):50.0ml

Final concentration	Stock concentration
0.1M EDTA	10ml (0.5M)
40% Sucrose	20g
25% Bromophenol blue	125mg

Final volume was made up to 50 ml with distilled water.

9) 10X Taq buffer:

Stock concentration	Vol./amt. taken	Final concentration
1M Tris-Cl (pH 6.8)	6.7 ml	0.67M
1M Ammonium sulphate	1.66 ml	0.166M
100% Triton X-100	0.45ml	4.5% (V/V)
BSA	20mg	0.2%

Final volume was made to 10 ml with HPLC grade sterile water.

Solution and buffers for PAGE:

1) Resolving gel buffer pH 8.9: Dissolved 18.15 gm Tris base in 60 ml distilled water. Adjusted the pH 8.9 by adding HCL and made the final volume 100 ml with water.

3) Stacking gel buffer: (0.6M Tris base pH 6.7) Dissolved 6.1g Tris base in 60 ml distilled water and adjusted pH6.7 by adding HCL and made up final volume 100 ml.

4)10% Ammonium per sulphate solution (APS): Dissolved 100 mg APS in 1ml distilled water.

5) Running electrode buffer (pH 8.3):

Tris Base	-	0.6g
Glycine	-	2.8g
Water	-	1 liter

6) Staining solution:

already mentioned on Page 44

Coomassie brilliant blue-R-250-		100 mg
Methanol	-	40 ml
Acetic acid	-	10 ml

Made up final volume upto 100 ml with D.water.

7. De-staining solution:

Methanol	-	40 ml
Acetic Acid	-	10 ml
Distilled Water	-	50 ml

Extraction buffers for isozyme and PAGE analyses:

0.05M Tris Base	-	0.605g
Sucrose (5%w/v)	-	5.0g
EDTA	-	168 mg
2-ME	-	100µl

Dissolved in 60 ml distilled water and adjusted the pH 7.5 by adding 1.0 N HCl and made up the final volume to 100 ml.

Reagent C (Alkaline copper solution): Mixed 50 ml of reagent 'A' and 1 ml of reagent 'B' prior to use.

(a) Reagent A: 2% Sodium carbonate in 0.1N Sodium hydroxide.

(b) Reagent B: 0.5% Copper sulphate ($CuSO_4$. $5H_2O$) in 1% Potassium sodium tartrate.

Alkaline copper tartrate:

(a) Dissolved 2.5 g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80 ml water and made up final volume to 100 ml.

(b) Dissolved 50 g copper sulphate in a small volume of distilled water. Added one drop of sulphuric acid and made up final volume to 100 ml.

Mixed 4 ml of B and 96 ml of solution A before use.

give under Lowry's method

PMSF (1M) stock: Dissolved 17.4 mg PMSF in 1.0 ml ethanol and stored in freeze.

2X Sample buffer for SDS PAGE:

Final concentration	volume taken	Stock
0.125M Tris HCl	0.125 ml	1M
Water	0.175 ml	
4% SDS	0.4 ml	10%
20% Glycerol	0.2 ml	100%
10% 2-ME	0.1 ml	100%
0.004% bromophenol blue	10 mg	

Final volume was made up to 1 ml with distilled water.

Electrode buffer for SDS PAGE:

Tris Buffer	3.0g
Glycine	13.3g
SDS	1g

Dissolved and made the final volume to 1 liter in water.

Sodium phosphate mono basic buffer (0.2M): Dissolved 27.8g of sodium phosphate in 200 ml and final volume made up to 1000ml with distilled water.

Sodium phosphate di-basic buffer (0.2 M): Dissolved 53.65g of dibasic sodium phosphate in 200 ml distilled water. Final volume made up to 1000 ml.

For pH 6.0, 87.7 ml mono-basic and 12.3 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.0, 39.0 ml mono-basic and 61.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

For pH 7.5, 16.0 ml mono-basic and 84.0 ml dibasic was mixed and made up to 200 ml with distilled water to get phosphate buffer of 100 mM.

Acetate buffer:

0.2M solution of acetic acid: 11.55 ml of acetic acid dissolved in 1000 ml of distilled water.

0.2M solution of sodium acetate: 16.4g of sodium acetate dissolved in 200ml-distilled water and made up the final volume 1000 ml.

For pH 5.0, 14.8 ml of acetic acid and 35.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.

For pH 5.6, 4.8 ml of acetic acid and 45.2 ml of sodium acetate was mixed and made to final volume to 100 ml to get the buffer of 100 mM.

Results

Assessment of morphological attributes, and cytological study in 30 *Dichanthium* accessions:

Morphological observations:

Variations in following qualitative and quantitative traits were observed among 30 *Dichanthium* accessions at 50% flowering stage (Table 2 and 3).

Habitat: All 25 north Indian accessions were erect type whereas accessions from South India were mixed type. Of these, 2 accessions were creeping type and 3 accessions were semi erect type

Stem type: Twenty three accessions possessed solid and 7 accessions possessed hollow type stem.

Leaf texture: In eleven accessions leaf texture was smooth while in 21 accessions it was rough.

Rachis hairiness: No hairs on rachis were observed in 26 accessions whereas in 4 accessions hairs were present.

Nodal hairs: Out of 30 accessions, 10 showed red nodes, whereas 9 and 11 accessions showed medium and less hairs respectively.

Nodal color: Out of 30 accessions, 11 showed dense hairs, whereas 9 and 11 accessions showed medium and less hairs respectively.

Spike color: Out of 30 accessions, 7 showed red colored spikes whereas 16 and 7 accessions showed light purple and light green spikes respectively.

Hairs on spike: Out of 30, no hairs were observed in 27 accessions while 3 accessions (IGBANG-D-2, IGKMD-10 and IGBANG-D-1) showed hairs on spikes.

Awns: Out of 30, awns were present in 9 accessions whereas 15 and 6 accessions showed medium and small awns respectively.

Pits on floret: Pits were absent in 29 accessions however pits was present in the florets of IGBANG-D-2.

Table 2: Morphological (Qualitative) data of 30 *Dichanthium* accessions observations at 50% flowering stage.

Accessions	Nodal color	Nodal hair	Spike color	Hair on spike	Awans	Leaf texture	Type of stem	Pits on floret	Hairs on rachis	Habitat
IG97-234	Red	Medium	Red	Present	Medium	Smooth	Solid	Absent	Absent	Erect
IG97-24	Red	Medium	Red	Present	Medium	Smooth	Solid	Absent	Absent	Erect
IG97-30	Red	Medium	Red	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-192	Red	Less	Red	Present	Medium	Smooth	Solid	Absent	Absent	Erect
IG97-247	Light Yellow	Dense	Light Purple	Present	Long	Smooth	Solid	Absent	Absent	Erect
IG97-241	Light Yellow	Medium	Light Purple	Present	Long	Rough	Solid	Absent	Absent	Erect
IG95-25	Light Yellow	Dense	Light Purple	Present	Long	Smooth	Solid	Absent	Absent	Erect
IG95-114	Light Yellow	Dense	Light Purple	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-147	Light Yellow	Medium	Light green	Present	Small	Rough	Solid	Absent	Absent	Erect
IG97-170	Light Yellow	Medium	Light green	Present	Long	Smooth	Solid	Absent	Absent	Erect
IG97-151	Light Green	Less	Light Purple	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-152	Light Yellow	Less	Light Purple	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-184	Light Yellow	Dense	Light green	Present	Medium	Smooth	Solid	Absent	Absent	Erect
IG97-158	Light Yellow	Medium	Light green	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-118	Light Green	Dense	Red	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-233	Light Yellow	Medium	Light Purple	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-189	Light Yellow	Less	Red	Present	Small	Rough	Solid	Absent	Absent	Erect
IG97-132	Red	Medium	Light green	Present	Long	Smooth	Solid	Absent	Present	Erect
IG97-218	Light Yellow	Dense	Light Purple	Present	Medium	Rough	Solid	Absent	Absent	Erect
IG97-130	Light Yellow	Dense	Light Purple	Present	long	Rough	Solid	Absent	Absent	Erect
IG97-121	Light Yellow	Dense	Light Purple	Present	Medium	Smooth	Hollow	Absent	Absent	Erect
IG97-244	Light Yellow	Less	Light Purple	Present	Small	Rough	Hollow	Absent	Present	Erect
IG97-245	Red	Less	Light Purple	present	Medium	Rough	Hollow	Absent	Present	Erect
IG97-144	Red	Dense	Light green	Present	Medium	Smooth	Solid	Absent	Absent	Erect
IGKMFD-1	Light Yellow	Less	Red	Present	Long	Rough	Solid	Absent	Absent	Semi erect
IGTGD-4	Red	Less	Light Purple	Present	Long	Rough	Hollow	Absent	Absent	Creeping
IGBANG-D-2	Light Yellow	Less	Light Green	Absent	Long	Rough	Solid	Present	Absent	Semi erect
IGKMD-10	Red	Less	Light Purple	Absent	Small	Smooth	Hollow	Absent	Absent	Semi erect
IG3108	Red	Less	Light Purple	Present	Small	Rough	Hollow	Absent	Absent	Creeping
IGBANG-D-1	Red	Dense	Light Purple	Absent	Small	Rough	Hollow	Absent	Present	Erect

Table 3: Morphological (Quantitative) data of 30 *Dichanthium* accessions at 50% flowering stage.

Accessions	Tussock height (m)	Internodal length (cm.)	Length of longest leaf (cm.)	Width of longest leaf (mm)	Number of nodes	Number of spikes per tiller	Number of spikelets per spike	Culm thickness (mm)
IG97-234	0.99 ±0.08	9.65 ±0.67	17.36	7	8	1	8-12	4
IG97-24	0.98 ±0.11	9.91 ±0.67	15.66	6	6	1	2	4
IG97-30	1.10 ±0.08	10.13 ±0.34	16.85	6	6	1	4	5
IG97-192	1.15 ±0.08	14.56 ±1.69	18.71	7	4	3	9	5
IG97-247	0.92 ±0.10	11.39 ±0.32	17.53	7	6	1	5	5
IG97-241	0.84 ±0.04	9.99 ±1.03	17.36	6	6	1	6	5
IG95-25	0.38 ±0.04	10.84 ±0.64	9.57	3	4	1	2	2
IG95-114	0.71 ±0.05	10.84 ±0.82	16.34	5	5	1	3	4.5
IG97-147	0.67 ±0.11	9.40 ±0.44	15.75	5	5	1	5	3
IG97-170	0.77 ±0.08	11.85 ±0.39	10.75	4	8	2	3	4
IG97-151	0.73 ±0.12	6.52 ±0.15	16.43	4	6	1	7	4
IG97-152	0.80 ±0.10	10.08 ±0.39	16.00	5	7	1	3	4
IG97-184	0.86 ±0.06	8.64 ±0.51	27.94	5	7	1	14	5
IG97-158	0.81 ±0.04	12.78 ±2.13	16.17	5	8	1	5	4
IG97-118	0.77 ±0.05	8.89 ±1.27	16.17	5	8	1	10	4
IG97-233	0.72 ±0.07	13.38 ±0.89	17.02	6	7	2	4	3
IG97-189	0.71 ±0.09	12.19 ±0.88	16.34	4	10	1	7	4
IG97-132	0.86 ±0.05	9.31 ±0.29	13.97	5	8	2	5	2.5
IG97-218	0.83 ±0.06	8.64 ±0.51	13.38	6	8	1	4	2
IG97-130	0.71 ±0.06	14.31 ±0.82	16.17	5	5	2	7	4
IG97-121	0.98 ±0.08	11.35 ±0.39	16.00	4	10	2	4	4
IG97-244	1.28 ±0.06	12.19 ±1.34	16.26	5	6	1	6	4
IG97-245	0.87 ±0.09	8.72 ±1.03	18.63	6	9	1	4	5.5
IG97-144	1.62 ±0.08	9.23 ±0.39	28.79	4	15	1	9	5.5
IGKMFD-1	1.37 ±0.09	11.68 ±0.25	26.25	6	9	1	7	7
IGTGD-4	1.27 ±0.12	11.35 ±0.39	19.64	5	14	1	3	6
IGBANG-D-2	0.81 ±0.04	12.11 ±0.96	23.37	5	5	1	7	6
IGKMD-10	0.78 ±0.06	9.06 ±0.39	16.34	5	8	1	2	6
IG3108	1.29 ±0.08	9.57 ±0.15	18.29	5	6	1	3	6
IGBANG-D-1	0.83 ±0.077	13.80 ±0.53	23.62	5	6	1		5.5
Mean	**0.91 ±0.07**	**10.74 ±0.688**	**17.75**	**5.11**	**7.33**	**1.23**	**5.43**	**4.45**

Tussock height: Among 30 *Dichanthium* accessions, it ranged from 0.38 (IG95-25) to 1.37m (IGKMFD-1) with an average tussock height of 0.91 meter.

Internodal length: It ranged from 5.52 (IG97-25) to 14.56 cm.(IG97-192) with an average of 10.74cm.

Leaf length of longest leaf: It ranged from 9.57 (IG95-25) to 28.79 cm. (IG97-144) with an average of 17.75cm.

Leaf width of longest leave: It ranged from 3.0 (IG95-25) to 7.0 mm. (IG97-234, IG97-192 and IG97-247) with an average of 5.11 mm.

Number of nodes per tiller: Among the accessions it ranged from 4-14 with an average of 5.11.

Number of spikes: It ranged from 1-3 with an average of 1.23.

Number of spikelets: It ranged from 3-12 with an average of 5.43.

Culm thickness: It ranged from 2.0 to 7.0 with an average of 4.45.

In comparison to control, under stress most drastically affected morphological traits observed were hairiness of leaves which decreased significantly. Advance in flowering in the tune of 10 to 15 days was observed.

Cytological observations:

All thirty accessions of *Dichanthium annulatum* revealed 2n=4x=40 chromosome number and characterized as tetraploid with normal disjunction (Fig. 2). Accessions revealed various kinds of chromosomal associations at diakinesis stage (Table 1). Out of 30, meiosis in 17 accessions was perfectly normal showed only bivalents accounts for 56.6 %, 12 accessions revealed single quadrivalent (42.33 %) at diakinesis and a accession IG97-130 gave 2 univalent along with 18 bivalents (18II+2I) formation at diakinesis. Interestingly most of accessions collected from south Indian plateau possesses only bivalents except accession IGBANG-D-2 having single quadrivalent (18II+1IV), whereas rest of accessions collected from north Indian plateau mixed chromosomal association *i.e.*, only bivalent and bivalent as well as quadrivalent was observed in PMC (Table 4).

Table 4: Agro-climatic regions and places of collections and chromosome association in individual accession of *Dichanthium* lines.

Accession no.	Agro-climatic regions of collection site	Chromoso --me no.	Chromosome association				Ploidy level	% of bivalent
			IV	III	II	I		
IG97-234	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-24	Lalitpur, north India	2n=40	1	0	18	0	Tetraploid	90
IG95-30	Lalitpur, north India	2n=40	0	0	20	0	Tetraploid	100
IG97-192	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-247	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-241	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG95-25	Lalitpur, north India	2n=40	1	0	18	0	Tetraploid	90
IG95-114	Lalitpur, north India	2n=40	0	0	20	0	Tetraploid	100
IG97-147	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-170	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-151	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-152	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-184	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-158	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-118	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-233	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-189	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-132	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-218	North India c. plateau	2n=40	0	0	20	0	Tetraploid	100
IG97-130	North India c. plateau	2n=40	0	0	19	2	Tetraploid	95
IG97-121	North India c. plateau	2n=40	1	0	18	0	Tetraploid	90
IG97-244	Chatterpur, north India	2n=40	0	0	20	0	Tetraploid	100
IG97-245	Chatterpur, north India	2n=40	0	0	20	0	Tetraploid	100
IG97-144	Chatterpur, north India	2n=40	0	0	20	0	Tetraploid	100
IGKMFD-1	Dharwad, south India,	2n=40	0	0	20	0	Tetraploid	100
IGTGD-4	Dharwad, south India	2n=40	0	0	20	0	Tetraploid	100
IGBANG-D-2	Bangalore, south India	2n=40	1	0	18	0	Tetraploid	90
IGKMD-10	Dharwad, south India	2n=40	0	0	20	0	Tetraploid	100
IG3108	Dharwad, south India	2n=40	0	0	20	0	Tetraploid	100
IGBANG-D-1	Bangalore, south India	2n=40	0	0	20	0	Tetraploid	100

IV- Quadrivalent , III-trivalent, II-Bivalent and I-Univalent, C. plateau = Central plateau

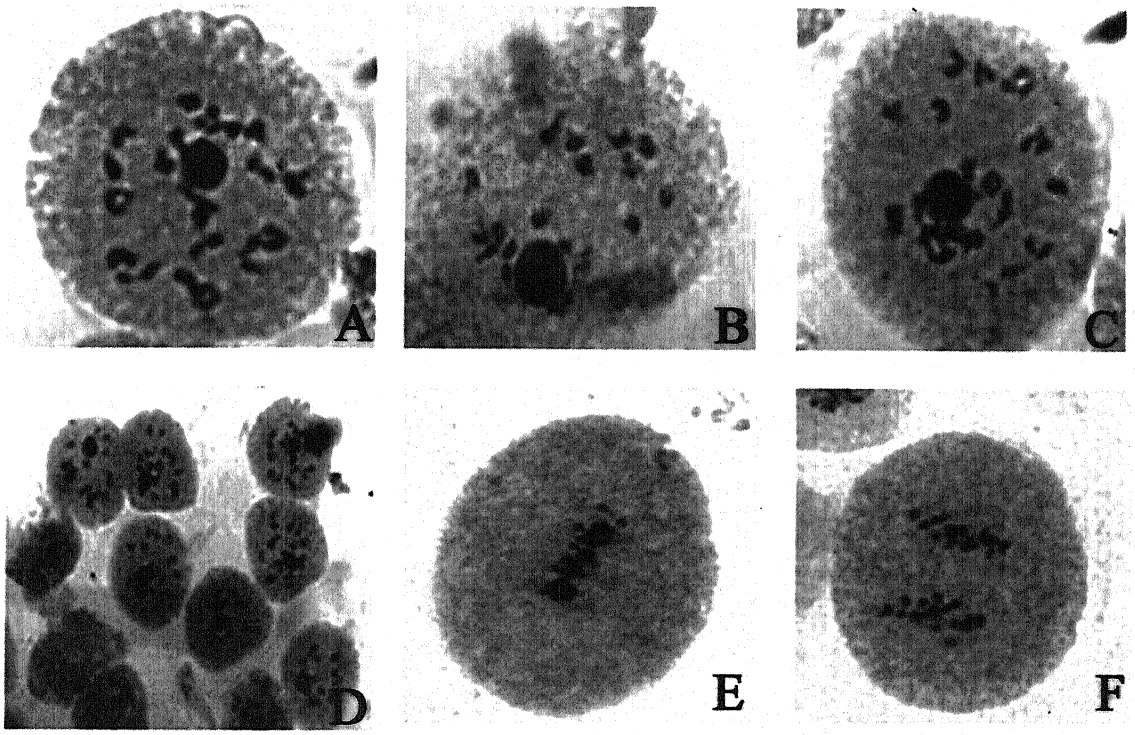


Fig.2. Stages of meiosis in *Dichanthium* genotypes. A, Diakinesis with 20II. B, Diakinesis with 20 II. C.Diakinesis with 18II+1IV.
D, Pollen mother cells at diakinesis stage. E, Metaphase as observed in ITGD-4 accessions. F, Anaphase with normal segeregation pattern.

Assessment of genetic diversity in *Dichanthium* genotypes using RAPD, STS, ISSR and Isozyme markers.

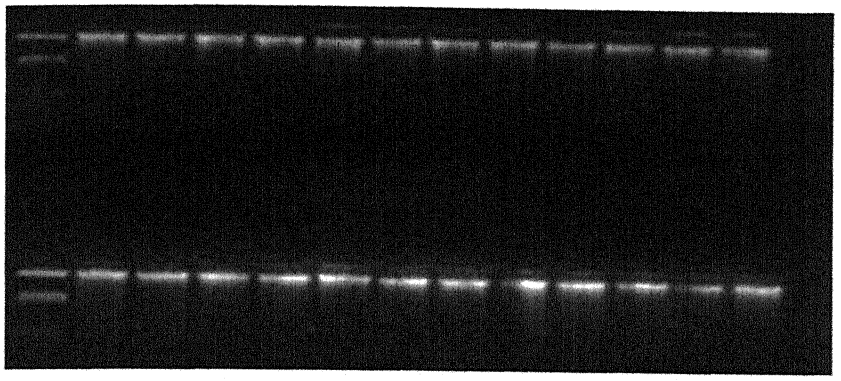
Four marker systems were used for estimation of genetic relationships among 30 *Dichanthium* accessions. Genomic DNA isolated from 30 accessions were digested with two restriction enzymes (EcoR1 and HindIII) and the results indicated complete digestion signifying the quality of DNA (Figure 3)

Identification and evaluation of RAPD primers for genetic diversity estimate in 30 *Dichanthium* genotypes:

RAPD analysis was carried out using 56 random decamer operon primers (Table 5). More than 200 primers from different series OPC, OPE, OPI, OPP, OPR, OPU of operon primers were screened to get best suitable primers, which gave clear and consistent reproducible amplified products. Out of 200 random primers screened 56 primers were finally chosen to develop the DNA fingerprints of 30 *Dichanthium* accessions (Figure 4 A, 4B and 4C). The selected primers represented the different series of operon. Only those amplified bands were scored which were consistent clear and reproducible. Very faint and ambiguous bands were omitted from scoring and therefore were not considered for further study. Out of 56 primers, the large fraction of total primers i.e., 23 primers produced only polymorphic bands exhibited 100% polymorphism where as 17 primers produced single monomorphic band exhibited an average of 92.82 % polymorphisms. Twelve primers produced two monomorphic bands with average 81.10% polymorphism and 4 primers produced more than 2 monomorphic bands with average 54.54% polymorphism. GC content of all the primers used ranged from 60-70%. The Tm value of the primers ranged from 34-32 °C.

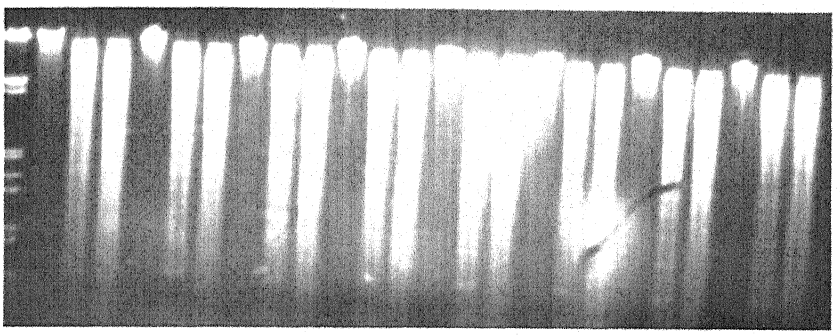
In total of 510 thick, clear, and unambiguous amplified bands were scored form 56 random primers with an average of 9.107 bands per primer. Out of 510 bands scored, 56 bands were monomorphic with an average of one monomorphic band per primer, and rest 454 bands were polymorphic with average of 8.107

M



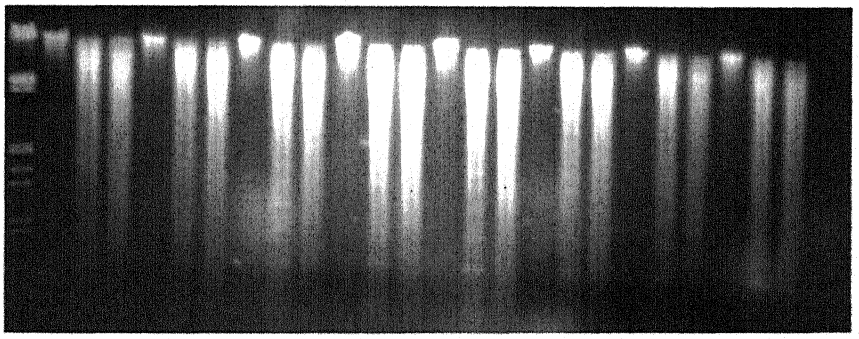
A

MUEH UEH UEH UEH UEH UEH UEH UEH



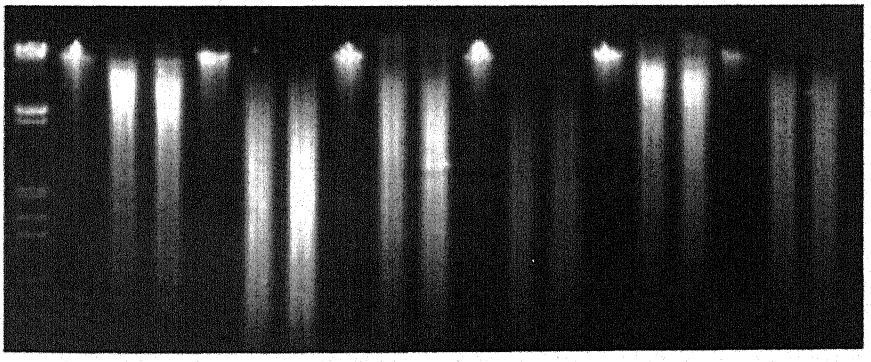
B

MUEH UEH UEH UEH UEH UEH UEH UEH



C

M U E H U E H U E H U E H U E H U E H



D

Fig.3. Genomic DNA isolated from Dichanthium genotypes M=λ Hind III digest (250 ng) (A). Genomic DNA digested with EcoR1 (E) and Hind III (H) restriction enzymes. U=uncut genomic DNA

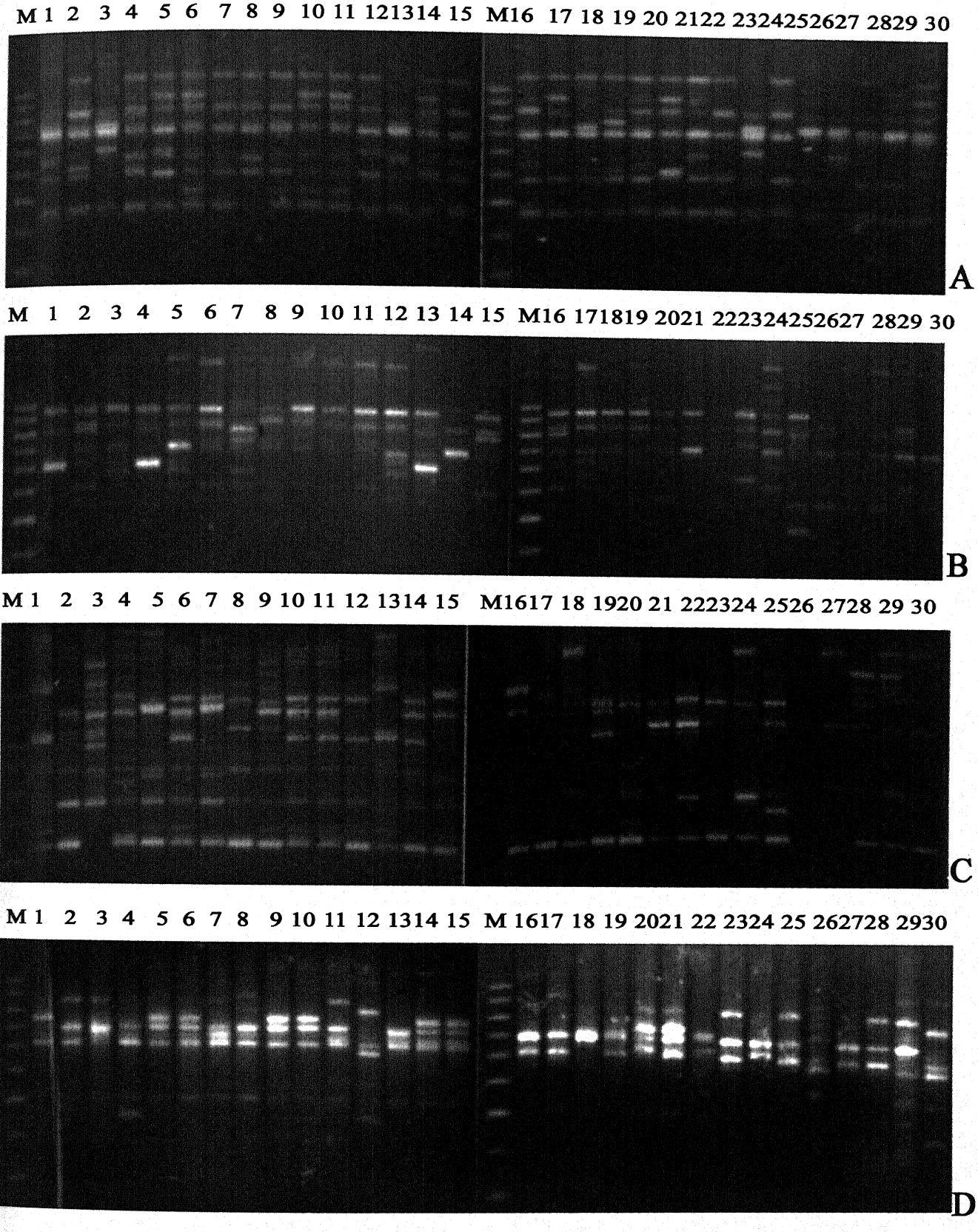


Fig.4A, RAPD profile with primers (A) OPF-06,(B) OPG-12,(C) OPP-9, (D) OPH-9 in 30 accessions of *Dichanthium*. M=100 bp ladder

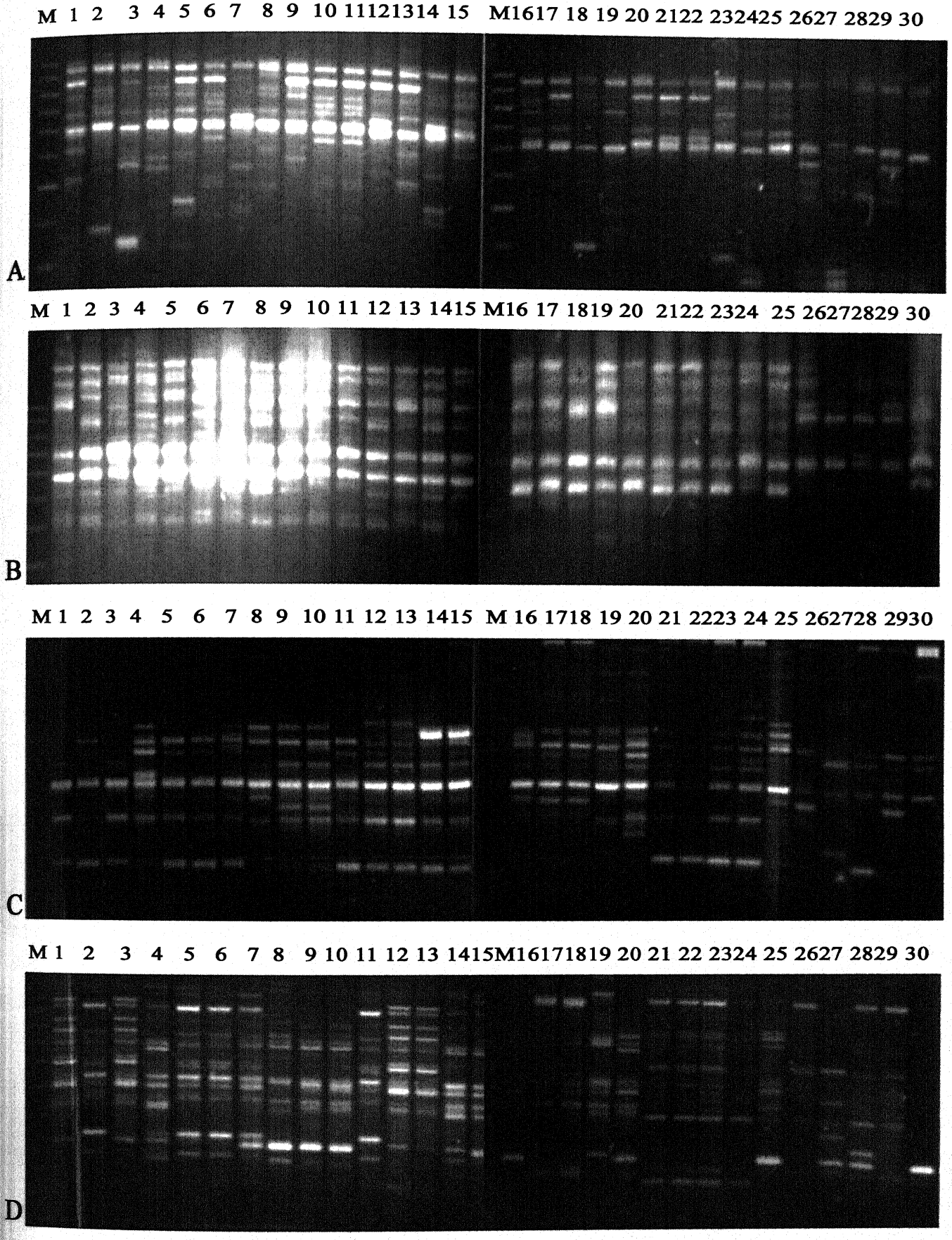


Fig. 4B. RAPD profile with primers (A)OPI-07, (B) OP E-15, (C) OPR-07, (D) OPR-08 in 30 accessions of *Dichanthium*. M= 100 bp ladder

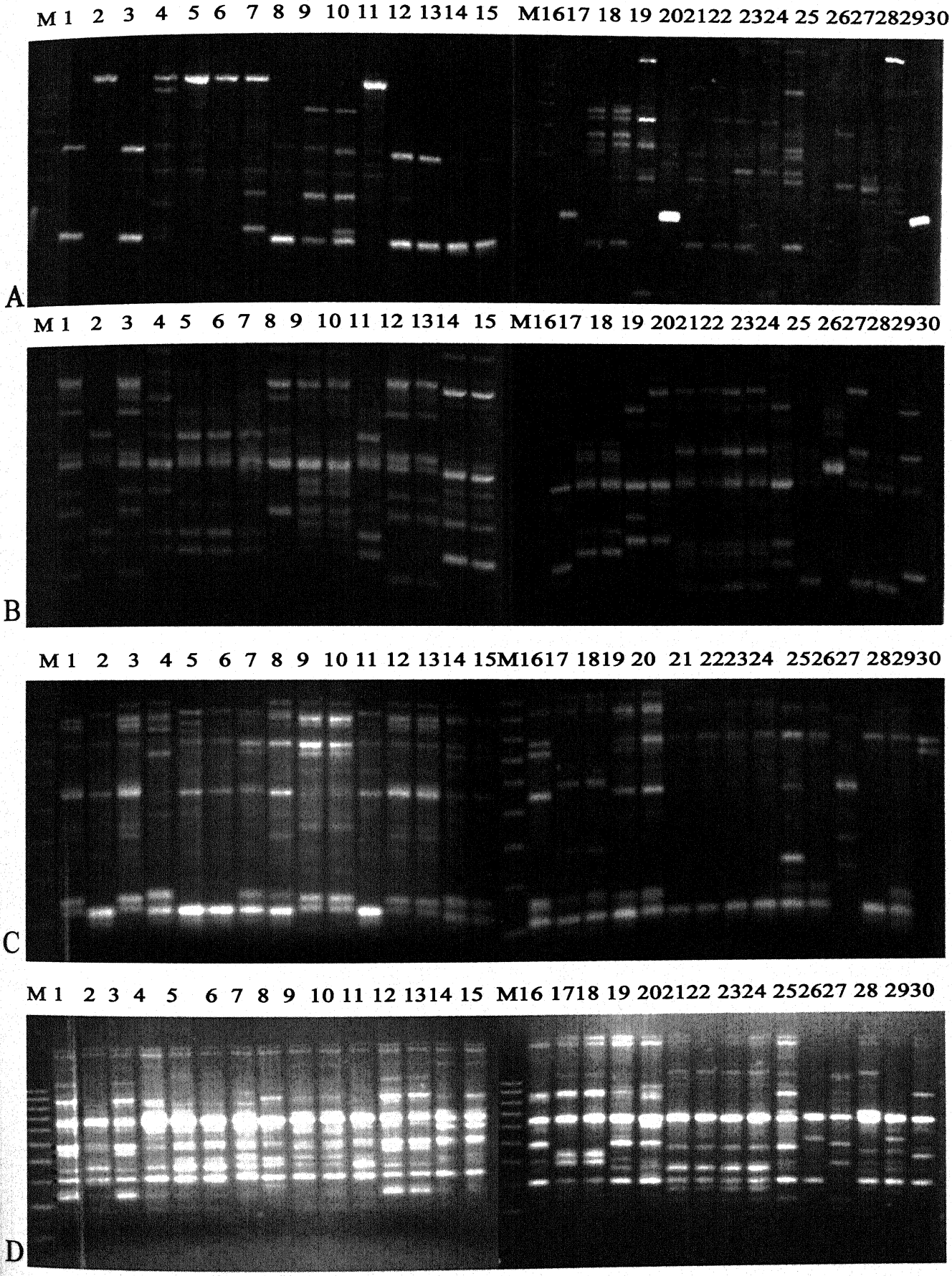


Fig. 4C. RAPD profile with primers (A)OPQ-05, (B) OPAB-01, (C) OPAH-07, (D) OPN-04 in 30 accessions of *Dichanthium*. M= 100 bp ladder

polymorphic bands per primer. High polymorphism of these primers makes them most suitable for genetic relationship and trait association studies, Among 56 primers, bands amplified by them ranged from 3 to 21 bands. Least number of amplified bands (3) was generated from primer OPP-7 and Maximum (21) bands were amplified from primer OPI-07, on an average 56 primers showed 88.51% polymorphism ranging from 42.86 to 100 %. Minimum polymorphism 42.86% was shown by primer OPC-02 where as maximum (100%) polymorphism shown 23 primers.

In total of 8945 amplified fragments were scored from 56 primers when 30 accessions of *Dichanthium* were taken into account. Of these 7265 amplified fragments were polymorphic and rest i.e., 1680 amplified fragments were monomorphic in nature. An average of 242.16 polymorphic bands (present) and 56 monomorphic alleles (presents) were scored per accession. These polymorphic bands were utilized to calculate the bands frequency, the polymorphic information content (PIC) and marker index (MI) to assess the resolving and discrimination power of primer among accessions. Polymorphic information content (PIC) of each individual primer was calculated to assess its ability to discriminate the accession, RAPD primers showed a high degree of PIC value, which further supports their suitability of genetic relationship and marker association study (Table 6). PIC value obtained from 56 primers ranged from 0.111 to 0.50 with an average of 0.448.The minimum PIC value i.e., 0.111 was obtained from primer OPI-11 where as maximum PIC value i.e., 0.5 was exhibited by set of four primers namely OPAH-01, OPF-01, OPAH-03 and OPAE-01 each. Except primer OPI-11 rest of 55 primers ~~were shown~~ showed higher average PIC value i.e., 0.454 (Table 5).

Marker index (MI) was obtained as the product of PIC and the number of polymorphic bands per assay. MI value obtained from 56 primers ranged from 0.44 to 7.33 with an average value of 3.702 (Table 5). The minimum MI value i.e., 0.44 was obtained from primer OPI-11 where as maximum MI value i.e., 7.33

Table 5: RAPD polymorphism produced from 56 primers in 30 *Dichanthium* accessions.

Primers	Sequences	GC (%)	Total bands (T)	Monomorphic bands (M)	Polymorphic bands (P)	Polymorphism (%)	PIC value	MI
OPC-02	5'GTGAGGCGTC3'	70	07	4	03	42.86	0.396	1.18
OPC-04	5'CCGCATCTAC3'	60	07	1	06	85.71	0.320	1.92
OPC-08	5'TGGACCGGTG3'	70	09	2	07	77.77	0.448	3.14
OPE-12	5'TTATCGCCCC3'	60	05	1	04	80.00	0.495	1.98
OPE-15	5'ACGCACAACC3'	60	09	4	05	55.50	0.448	2.24
OPE-16	5'GGTGACTGTG3'	60	08	-	08	100.0	0.370	2.96
OPI-11	5'ACATGCCGTG3'	60	04	-	04	100.0	0.111	0.44
OPP-07	5'GTCCATGCCA3'	60	03	-	03	100.0	0.320	0.96
OPQ-05	5'CCGCGTCTTG3'	70	12	-	12	100.0	0.398	4.78
OPQ-09	5'GGCTAACCGA3'	60	12	1	11	91.66	0.499	5.49
OPR-04	5'CCCGTAGCAC3'	70	13	1	12	92.30	0.467	5.14
OPR-05	5'GACCTAGTGG3'	60	08	1	07	87.50	0.499	3.49
OPR-06	5'GTCTACGGCA3'	60	06	-	06	100.0	0.499	2.99
OPU-02	5'CTGAGGTCTC3'	60	09	1	08	88.88	0.482	3.85
OPU-04	5'ACCTTCGGAC3'	60	07	-	07	100.0	0.457	3.19
OPU-06	5'ACCTTTGCGG3'	60	12	1	11	91.66	0.496	5.46
OPU-08	5'GGCGAAGGTT3'	60	08	3	05	62.50	0.497	2.98
OPAB-03	5'TGGCGCACAC3'	70	11	2	09	81.81	0.480	3.84
OPAB-05	5'CCCGAAGCGA3'	70	09	-	09	100.0	0.491	4.43
OPAE-03	5'CATAGAGCGG3'	60	10	2	08	80.00	0.480	3.84
OPAL-05	5'GACTGCGCCA3'	70	09	4	05	55.55	0.468	2.34
OPAL-12	5'CCCAGCCTAC3'	70	06	1	05	83.33	0.497	2.48
OPAL-14	5'TCGCTCCGTT3'	60	13	1	12	92.30	0.488	5.86
OPAL-16	5'CTTTCGAGGG3'	60	06	2	04	66.66	0.236	0.94
OPAH-01	5'TCCGCAACCA3'	60	07	1	06	85.71	0.500	3.00
OPE-01	5'CCCAAGGTCC3'	70	10		10	100.0	0.487	4.87
OPE-08	5'TCACCACGGT3'	60	7	-	7	100.0	0.464	3.25
OPF-01	5'ACGGATCCTG3'	60	12	2	10	83.3	0.500	5.00
OPF-04	5'GGTGATCAGG3'	60	7	1	6	85.71	0.206	1.24
OPF-06	5'GGGAATTCGG3'	60	10	-	10	100.0	0.482	4.82
OPG-02	5'GGCACTGAGG3'	70	6	-	6	100.0	0.499	2.99
OPG-12	5'CAGCTCACGA3'	60	5	-	5	100.0	0.496	2.48
OPH-04	5'GGAAGTCGCC3'	70	8	1	7	87.50	0.296	2.07
OPH-05	5'AGTCGTCCCC3'	70	7	-	7	100.0	0.448	3.14
OPH-09	5'TGTAGCTGGG3'	60	10	-	10	100.0	0.481	4.81
OPH-13	5'GACGCCACAC3'	70	9	-	9	100.0	0.462	4.16
OPI-07	5'CAGCGACAAG3'	60	21	2	19	90.47	0.377	7.18
OP-I08	5'TTTGCCCGGT3'	60	8	1	7	87.50	0.485	3.40
OPI-14	5'TGACGGCGGT3'	70	10	-	10	100.0	0.497	4.97
OPI-18	5'TGCCCAGCCT3'	70	6	1	5	83.33	0.468	2.81
OPAB-01	5'GAGCGCCTTG3'	70	14	-	14	100.0	0.495	6.42
OPAE-04	5'CCAGCACTTC3'	60	8	2	6	75.00	0.492	2.95
OPAE-07	5'GTGTCAGTGG3'	60	15	2	13	86.66	0.474	6.16
OPAH-03	5'GGTTACTGCC3'	60	11	-	11	100.0	0.500	5.50
OPAH-09	5'AGAACCGAGG3'	60	10	-	10	100.0	0.491	4.91
OPB-05	5'TGCGCCCTTC3'	70	11	-	11	100.0	0.443	4.87
OPN-04	5'GACCGACCCA3'	70	9	2	7	77.77	0.487	3.41
OPN-06	5'GAGACGCACA3'	60	8	1	7	87.50	0.460	3.24
OPP-09	5'GTGGTCCGCA3'	70	8	-	8	100.0	0.438	3.57
OPQ-06	5'CCGTCGGTAG3'	70	12	1	11	91.66	0.472	5.19
OPR-07	5'ACTGGCCTGA3'	60	13	1	12	92.30	0.483	5.19
OPR-08	5'CCCGTTGCCT3'	70	15	-	15	100.0	0.488	7.33
OPAE-01	5'TGAGGGCCGT3'	70	10	2	8	80	0.500	5.00
OPF-08	5'GGGATATCGG3'	60	6	2	4	66.66	0.433	1.731
OPAH-08	5'TTCCCGTGCC3'	70	10	2	8	80	0.489	3.919
OP-06	5'GTGGGCTGAC3'	70	4	-	4	100	0.459	1.839
Total			**510**	**56**	**454**			
Mean		**60.70**	**9.107**	**1.00**	**8.107**	**88.51**	**0.4481**	**3.702**

was obtained from primer OPR-08. Two primer namely OPAB-01 and OPAE-07 exhibited higher MI value i.e., 6.42 and 6.16 respectively. Among the 56 random RAPD primer, OPI-11 was least polymorphic and informative because of its low PIC (0.111) and corresponding MI (0.44) value (Table 5).

Genetic diversity and clustering pattern of 30 *Dichanthium* genotypes based on RAPD polymorphism data:

Pair wise genetic distance was generated based on Dice similarity coefficient of 510 RAPD bands. Dendrogram was constructed using SHAN clustering using the UPGMA algorithm; three main clusters were obtained namely cluster I, cluster II and cluster III at 72.61% genetic similarity level which together embodied 29 *Dichanthium* accessions. One accession i.e., IGBANG-D-2 from south India, was observed to be separated from other accessions at 55.13% similarity level. Cluster II is separated from cluster I and Cluster III at 72.61 and 67.84% genetic similarity level respectively (Figure 5).

Cluster I. included 4 accessions namely, IG 97-234, IG 97-152, IG 97-184 and IG 95-30. The Intra- cluster range of cluster I ranged from 84.76 – 89.5%. All four accessions were collected from northern India, these accessions together showed 84.76% genetic similarity. Accessions IG 97-15 and IG97-184 showed maximum genetic similarity (89.5%) in this group. These two accessions shared (86.2%) genetic similarity with accession IG 97-234,where as accession IG 95-30 shared 84.76 % genetic similarity with rest of the accessions cluster. Intra- cluster range of cluster I ranged from 84.76 – 89.5% genetic similarity.

Cluster II. Among the three clusters cluster II was the largest cluster consisted of 21 *Dichanthium* accessions. It accounts to 70% of total accessions used for genetic diversity study. Accessions of this cluster shared the wide range of intra-cluster variability in genetic similarity ranged from 75.0 to 95.36%. This indicated that all 21 accessions of this cluster shared minimum 75% genetic similarity.

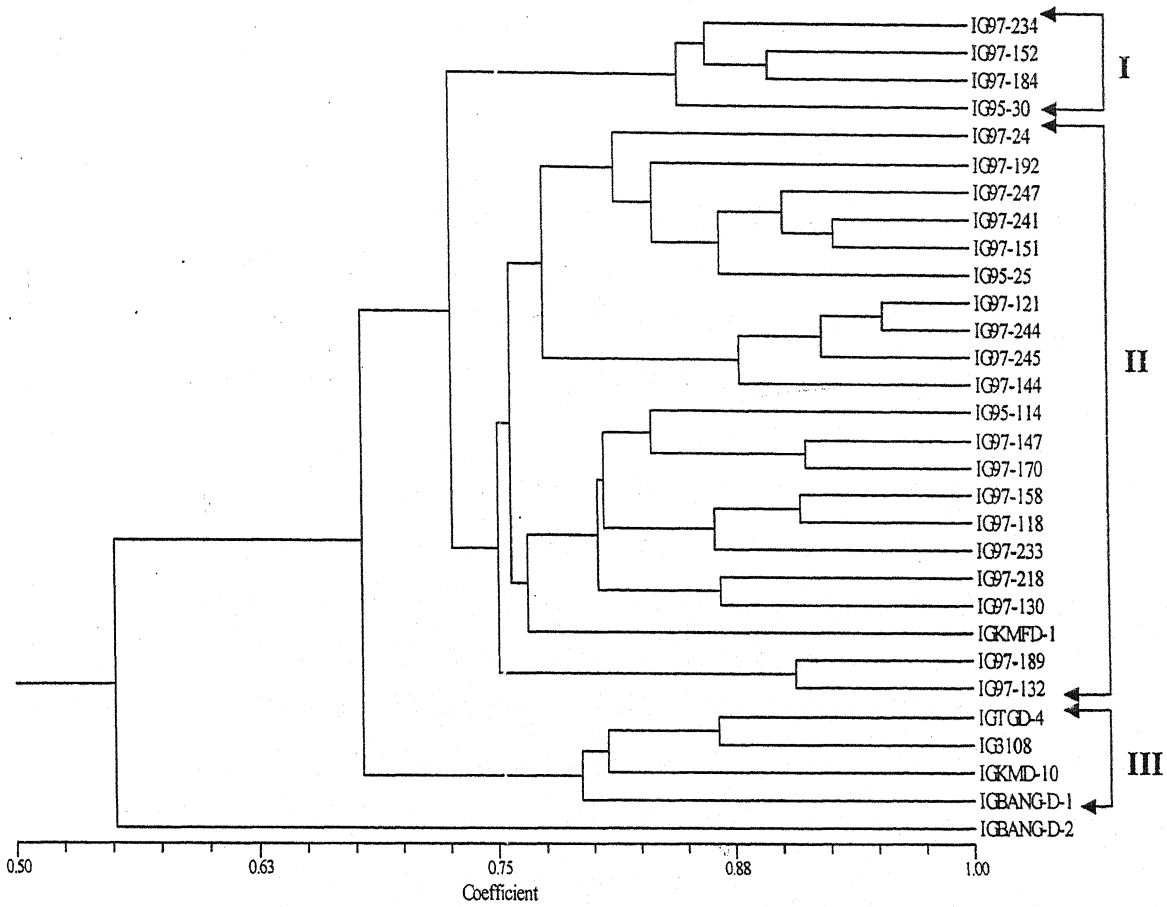


Fig. 5. Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on 510 RAPD bands.

Out of 21 *Dichanthium* accession of this group, 20 accessions were belonged to north Indian region but one accession namely IGKMFD-1, belonged to south India. Cluster II was further subdivided into three subclusters namely subcluster IIA, IIB and IIC. Subcluster IIA and IIB shared (75.73 %) genetic similarity. Subcluster IIA, IIB and IIC included 10, 9 and 2 accessions respectively. Subcluster IIC joined IIA and IIB at 75.01 % similarity level.

Subclusters IIA. Embodied 10 accessions which shared 77.54% genetic similarity. The Intra cluster variability of IIA ranged from 77.54 to 95.36%. Within this subcluster IIA, a group of 4 accessions namely IG 97-247, IG97-241, IG97-151 and IG 95-25 showed more (86.93 %) genetic similarity. Out of 4 accessions, 2 accessions i.e., IG 97-241 and IG 97-151 exhibited maximum (92.91%) genetic similarity with each other. These accessions further shared genetic joined the accessions IG 97-247 and IG 95-25 at 90.23 and 86.93% genetic similarity level respectively. Within subcluster IIA, an another group of 4 accessions namely IG 97-121, IG 97-244, IG 97-245 and IG 97-144 shared (87.88%) genetic similarity with each other. Among these 4, 2 accessions viz., IG 97-121 and IG 97-244 shared maximum 95.36% genetic similarity. Further these accessions joined rest of two accessions of this group namely IG 97-245 and IG 97-144 at 92.17 and 87.88% genetic similarity respectively. Other 2 accessions of subcluster IIA namely IG 97-24 and IG 97-192 joined rest of the accessions of IIA at 81.3 and 83.3 % genetic similarity respectively.

Subcluster IIB. Consisted 9 accessions, namely IG 97-114, IG 97-147 , IG 97-170, IG 97-158, IG 97-118, IG 97-233, IG 97-218, IG 97-130 and IGKMFD-1.These accessions shared minimum (76.57%) genetic similarity with each other. Intra cluster variability in genetic similarity of subcluster IIB was ranged 76.57 to 91.36 %. Among the accessions of IIB only single accession namely IGKMFD-1 belonged to south India while rest of the accessions was from central plateau and north India. Out of nine 8 accessions of subcluster IIB shared genetic similarity ranged from 80.31 to 91.36%. Accession IGKMFD-1 joined these accessions at 76.57% similarity. This subcluster contained two accessions namely IG 97-147 and IG 97-170 showed maximum (91.36%) genetic similarity where as accession

IG95-144 joined these accessions at 83.22% genetic similarity. Another set of 2 accessions namely IG 97-158 and IG 97-118 exhibited close (91.02%) genetic similarity; however these accessions shared 86.50% genetic similarity with accession IG 97-233, which further shares 80.67 genetic similarity with other accessions of this sub cluster IIB. A set of two accessions namely IG 97-218 and IG 97-130 shared high (86.83%) genetic similarity and diverged from rest of the accessions of subcluster IIB at 80.31% genetic similarity.

Subcluster IIC. A small subcluster contained only two accessions namely IG 97-189 and IG 97-132 both from north India. Both the accessions exhibited close genetic relationship shared 90.70% genetic similarity. This subcluster further joined to IIA and IIB at 75.01% genetic similarity.

Cluster III. A small cluster embodied 4 accessions namely IGTGD-4, IG3108, IGKMD-10 and IGBANG-D-1. Accessions of this cluster shared minimum 79.43% genetic similarity. Accessions included in this cluster belonged to south India. Cluster III separated other clusters at low similarity level, which exhibited (67.84%) genetic similarity with other clusters. The accessions of this cluster shared the genetic similarity ranged (intra cluster variability) from 79.43 to 86.84%. Two accessions i.e., IGTGD-4 and IG 3108 shared 86.64% genetic similarity with each other. Accession IGKMD-10 and IGBANG-D-1 joined with these two (IGTGD-4 and IG 3108) at 80.79% and 79.43% genetic similarity respectively.

Evaluation of sequence tagged sites (STS) primers for diversity estimate in 30 *Dichanthium* genotypes:

In total 17 sequence tagged sites (STS) primers (reverse and forward primer) were used to develop DNA finger prints, these primers were derived from were derived from the pst1clones obtained from *S. scabra* cv Fitzroy and *S.hamata* cv Verano as well as different non-coding and coding region of gene (Table 6).

All 17 Primers were tested at three different annealing temperatures. i.e., 52°C, 55°C and 58°C for assessing the most appropriate annealing temperature

Table 6: Description of sequence tagged-sites (STS) loci in 30 *Dichanthium* accessions.

STS primer pairs	Sequences	Target region	Intron	Microsatellites motif , if any	Total bands	Poly. bands	% poly.	PIC	MI	Ann. Temp.	Ref No.
S HCADIAF2 SHCADIAR2	5' GCTTAGGCCATGTTGCCATC 3' 5' ATCTCCTGAGTCTCCTTCAG 3'	C	Yes	(TTA)	4	4	100	1.68	0.420	52°c	Vander, *et al*,. 1999.
SHST3F3 SHST3R3	5' GGTTAACATAATAAAGCATG 3' 5' GTCTTGTAAACAATTCCAAGC 3'	C	Yes	(TAT), (T), (GATTTC), (A)	10	9	90.0	4.49	0.499	52°c	Smith, *et al.*, 1995.
SHPALF2 SHPALR2	5' TTCACGGCAATGTGCCAAGG 3' 5' AGGTATTGTATCTGTGCCCA 3'	NC	Yes	(TTA), (A)	11	8	72.72	3.99	0.499	52°c	Manners, *et al.*, 1995
SHCAPEAF1 SHCAPEAR1	5' TAATGTTGTGTCTTGTGCTG 3' 5' GCTGCTCAAAAGCTGACAAC 3'	C	Yes	(CTAA)	9	7	88.99	3.42	0.488	52°c	Reddy, *et al.*, 1996
SHST1F1 SHST1R1	5' GAAGCAACTCTTCTTCACAT 3' 5' GGCTTGTCAGGGAAGAAAGT 3'	C	No	(GAG)	5	5	100.0	2.95	0.491	52°c	Smith, *et al.*, 1995.
SHST3F1 SHST3R18	5' TAACTCTTGCCAGCCTCA 3' 5'CTGCACCTGCCATGAATCCCAC 3'	C	Yes	(T), (A), (TAT)	8	6	75.0	2.93	0.489	52°c	Smith, *et al.*, 1995.
SHST2F3 SHST2R16	5' AAGAACAAGAAACTCTTCTGG 3' 5'CCATGTAGTTCACTGCTGACCGAG 3'	C	Yes	(ATAA), (TAT)	9	6	66.66	2.89	0.482	52°c	Smith, *et al.*, 1995.
SHCAPEAF1 SHCAPEAR12	5' TAATGTTGTGTCTTGTGCTG 3' 5'TAGCCCATCTCTGCGTCC3'	NC	Yes	-	10	6	60.0	2.18	0.363	52°c	Vander, *et al.*, 1999.
SHCAPEAF11 SHCAPEAR1	5'GCCTCAACACAAAAGACCTTG3' 5' GCTGCTCAAAAGCTGACAAC 3'	NC	Yes	-	8	7	87.50	1.63	0.233	52°c	Vander, *et al.*, 1999.
SsCS247P1 SsCS247P2	5' CCAGATTGGGTTCGGATTCG 3' 5' GAGAAACAGATGGCATCAGA 3'	-	-	-	5	4	80.0	1.73	0.433	52°c	Liu, *et al.*, 1996
SsCS268P1 SsCS268P2	5' CAGCGGGTGGAGAAAAGAAG3' 5'AGGAACAAGTGCTGAAGAATAATG 3'	-	-	-	7	6	85.71	2.97	0.495	52°c	Liu, *et al.*, 1996
SsCS284P1 SsCS284P2	5' TCCGAAAAACCAGACACAGG 3' 5' AAGGTCTGCCATGGTATTGT 3'	-	-	-	6	5	83.33	2.44	0.489	52°c	Liu, *et al.*, 1996
SsCS4P1 SsCS4P2	5' ACAAGGTCCAAGAGAGCAAC 3' 5' ACATTTCTTTCCTCCACAGC 3'	-	-		6	5	80.00	1.82	0.364	52°c	Liu, *et al.*, 1996
SsCS15P1 SsCS15P2	5' GGTCCTCCAATAGAAACTGC '3 5' GCTACCTGGGCTTTTGGC 3'	-	-	-	8	6	75.00	1.33	0.222	52°c	Liu, *et al.*, 1996
Total					106	84					
Mean					7.57	6.0	80.10	2.60	0.426	52°c	

C= coding, NC= non-coding, NR= no reaction

(best amplification reaction), result showed that primers worked best at 52°C Annealing temperature (Figure 6A and B). Out of 17 STS primers, only 14 performed well and gave amplification reaction at 52°C (Table 6). Three primers namely SsCS71P1-SsCSP2, $SHST_1F3$-$SHST_1R3$ and SHCAPEF3-SHCAPER3 gave no amplification reaction at all at any of three annealing temperature tested with *Dichanthium* accessions. Total number of bands generated from 14 STS primers were ranged from 4 to 11 with average of 7.57 bands per primer, where as polymorphic bands were ranged from 4 to 9 with average of 6.0 bands per primer which accounts of average 80.1 % polymorphism, it was ranged from 66.66 to 100%. Among 14 selected working primers, 2 primers i.e., T_1F_1/T_1R_1 and IAF_2/ IAR_2 produced no monomorphic bands and thus exhibited maximum (100%) polymorphism, where as rest of 12 primers generated monomorphic bands ranged from 1 to 4 (Table 6).

In total 106 amplified bands were scored from 14 primers based on their clarity and reproducibility. Of these 84 bands were polymorphic and 22 bands were monomorphic. The pooled data obtained form 14 primers an average 7.57 bands were scored per primer. Out of which 6.0 bands per primer were polymorphic and 1.57 bands per primer were monomorphic. Among the 14 STS primers, the maximum (11) bands were obtained with primer pair ALF_2/ALR_2, where as second highest 10 bands with primer pairs AR_{12}/AF_1 and T_3F_3/T_3R_3 each. The minimum (4) bands were obtained from primer pair $1AF_2/1AR_2$. Though less number of monomorphic bands were scored, maximum 4 monomorphic bands out of 10 bands were obtained with primer AR_{12}/AF_1 which exhibited least polymorphism i.e., 60.0%. To assess the suitability and discrimination ability of STS primers polymorphic information content (PIC) and Marker index (MI) were obtained. Polymorphic information content (PIC) value obtained from each individual primer with *Dichanthium* accessions was ranged from 0.222 to 0.499 with an average of 0.426 per primer pair. The maximum (0.499) PIC value obtained from primer pairs namely ALF2-ALR2 and T_3F_3-T_3R_3, where as minimum (0.222) was obtained from $15P_1/15P_2$ (Table 6).

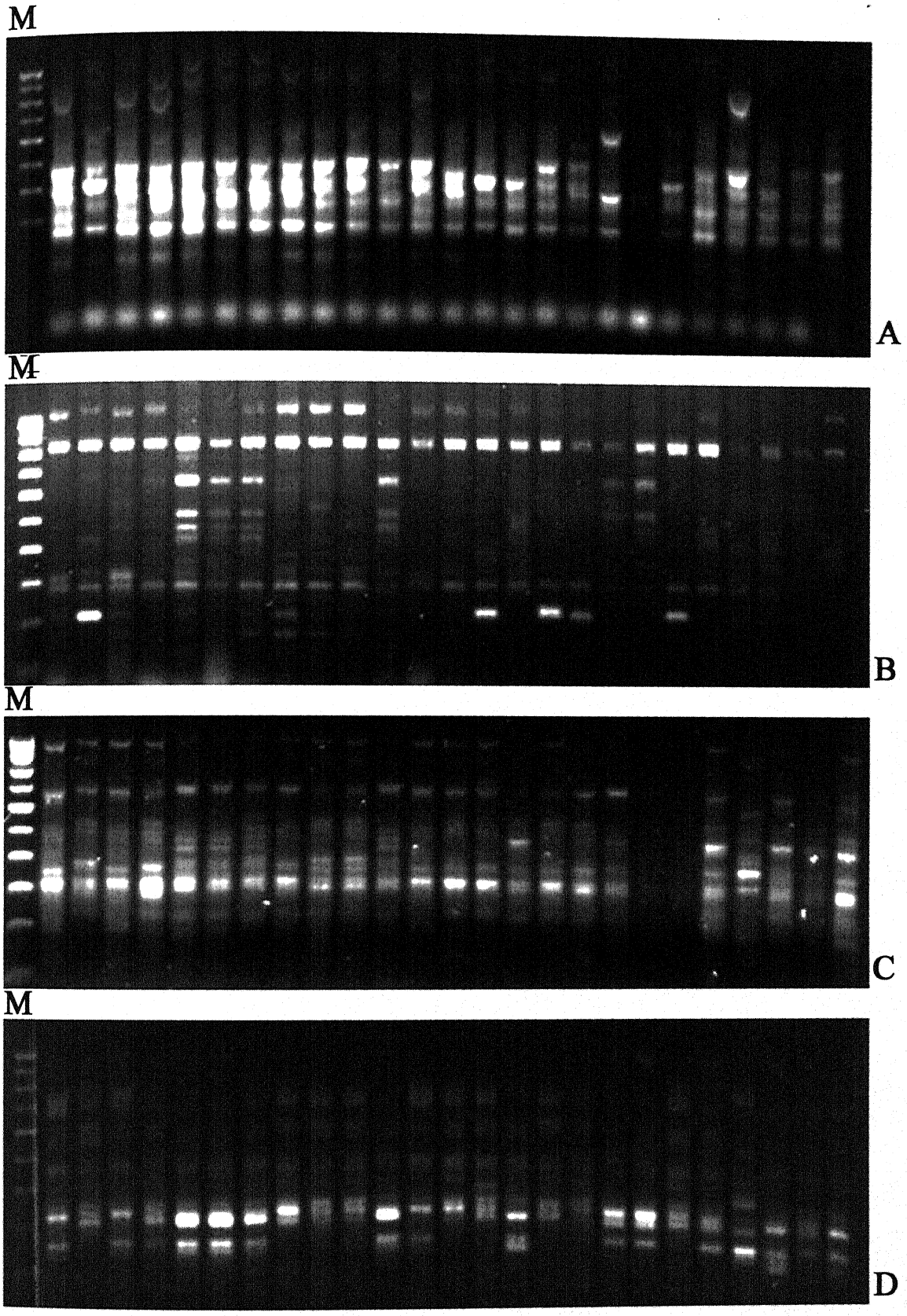


Fig. 6A. STS profile with primers (A) 268P1/268P2, (B) T2F3/T2R3, (C) 284P1/284P2, (D) 4P1/4P2, in accessions of *Dichanthium*. M= 100 bp ladder

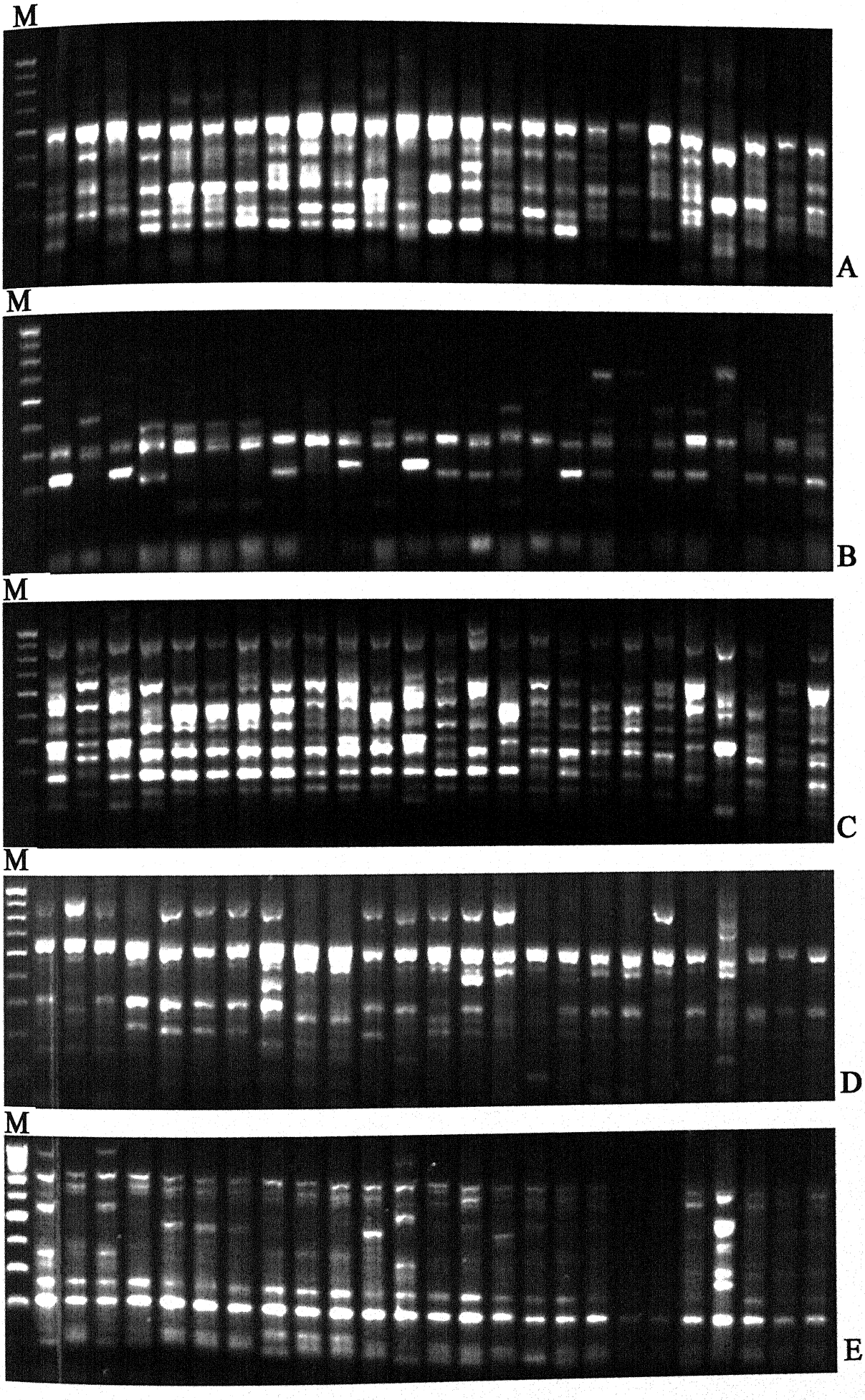


Fig. 6B. STS profile with primers (A) T3F1/T3R18, (B) T2F3/T2R16, (C) 15P1/15P2, (D) ALF2/ALR2, (E) AF1/AR1 in accessions of *Dichanthium*. M= 100 bp ladder

Out of 14 STS primers pairs 8 exhibited PIC values ranged from 0.482 to 4.99. Primer pairs 247 P_1/P_2 and $1AF_2/R_2$ exhibited PIC value i.e., 0.433 and 0.420 respectively. Four primers namely $15P_1/15P_2$, T_3F_3/ T_1R_1, AR_{12}/AF_1 and $4P_1/4P_2$ showed PIC values ranged from 0.222 to 0.364 viz., 0.222, 0.233, 0.363 and 0.364 respectively. Marker index (MI) values obtained from these primers were ranged from 1.33 to 4.49. An average MI value i.e., 2.60 was obtained using this marker system. The maximum MI (4.49) value was recorded in primer $71P_1/71P_2$ where as minimum MI value (1.33) in primer 15 $P_1/15P_2$.Among these 6 primers showed in the range of 2.18 to 2.97 five primers in the range of 1.336 to 1.824 whereas and rest three primers namely ALF_2/ALR_2, AF_1/AR_1 and T_3F_3/T_3R_3 showed MI values of 3.99, 3.419 and 4.49 respectively (Table 6).

Assessment of genetic diversity and clustering pattern of 30 *Dichanthium* accessions based on STS polymorphism data:

The estimation of genetic relationship and clustering pattern of 30 *Dichanthium* accessions was assessed using dendrograms generated from data obtained from 14 STS primers. Based on 106 amplified STS bands, a pair wise genetic distance was calculated in term of Dice similarity coefficients. Dendrogram was constructed by sequential agglomerative hierarchical and nested (SAHN) clustering using the Un-weighted pair group method with arithmetic mean (UPGMA) algorithm. Only the clear, consistent and reproducible bands were scored in all the accessions for each primer for dendrograms generation and data analysis. Dendrogram generated based on STS marker revealed the 3 major clusters namely cluster I, cluster II and cluster III (Figure 7). These clusters were separated at 75% genetic similarity level. Out of 30 , 29 accessions were grouped in various clusters except one accession i.e., IGBANG-D-2. Accession (IGBANG-D-2) was belonging to south India and separated from rest of the accessions at 70% genetic similarity level.

Cluster I: It is largest cluster, consisted of 21 accessions which shared 70% of total accessions. The intra cluster variability for similarity was ranged from 79 to 100%. Accessions of this cluster shared minimum 79% genetic similarity. Cluster

I joined cluster II at 75% genetic similarly, where as cluster III joined cluster I and II at 74% genetic similarity level. Cluster I embodied of accessions collected from northern and central plateau region of India. Cluster I was further subdivided into two subclusters namely subcluster IA and subcluster IB. Subcluster IA and IB joined each other at 79% genetic similarity level. Subcluster IA consisted of 17 accessions and subcluster IB includes 4 accessions.

Subcluster IA: It is a largest subcluster embodied 17 accessions, which were further clustered into three small groups namely group I, II and III. These groups include 4, 8 and 5 accessions respectively. Intra cluster variability for similarity in this subcluster was ranged from 79 to 100%.

Group1. Included 4 accessions namely IG97-234, IG95-30, IG 97-152 and IG 97-184, shared minimum 91% genetic similarity among each other. Accessions IG97-234 and IG95-30 shared 97 % similarity and joined other 2 accessions of the group at 91% similarity. Accession i.e., IG 97-152 and IG 97-184 shared 100% genetic similarity hence occupied the same position in dendrogram. This group exhibited intra-cluster similarity ranged from 91 to 100%

Group 2. This group included 8 accessions and shared 85% genetic similarity. Group II joined group1 at 81% genetic similarity level. This group also contained 2 accessions, namely IG 97-118 and IG97-233 exhibited 100% genetic similarity with each other and belonging to north India central plateau, further shared 87% genetic similarity with accession IG97-158 of the same group. Accessions namely IG 97-192 and IG 95-114 shared 87% genetic similarity and joined other accessions of this group at 85% genetic similarity. Together these 5 accessions namely IG 97-192, IG 95-114, IG97-158, IG 97-192 and IG 95-114 forms a subset and shared 86% genetic similarity. Two accessions namely IG 97-147 and IG 97-170 of the group shared 95.0 % genetic similarity and exhibited a strong closeness as indicated by boot strap value i.e., 95.3. These accessions joined IG97-24 at 86% similarity level. In this group intra-cluster similarity was ranged from 85 to 100%. Accessions of this group formed bridge between group1 and group 3 and shared 81% genetic similarity with Group1 and 79% with Group 3 respectively.

Group 3: It consisted of 5 accessions. Accessions of this group shared minimum 83% genetic similarity. This group joined other groups within the cluster II at 80% genetic similarity. Intra-cluster variability of similarity was ranged from 83 to100%. A set of three accessions namely IG97-121, IG97-244 and IG97-245 of this group exhibited 100% genetic similarity and occupied the identical position in dendrograms boot strap value i.e., 100% at their joining node also indicated their strong genetic relationship. These accessions shared 83% genetic similarity with other two accessions of the group namely IG97-218 and IG97-130, these accessions namely IG97-218 and IG97-130 showed strong association as indicated by boot strap value i.e., 68.1% at their joining node to each other. Further these 2 accessions together shared 90% similarity.

Subcluster IB: Possessed of 4 accessions (IG 97-247, IG 95-25, IG 97-241 and IG-97-151) with intra cluster genetic similarity ranging from 90-98%. Two accession namely IG 97-247 and IG 95-25 shared 98% genetic similarity and joined accession IG 97-241 at 96% similarity level, whereas accession of this subcluster i.e., IG-97-151 joined these accessions at 90% similarity level. Subcluster IB joined to subcluster IA at 79% similarity level. The grouping of accessions in this subcluster showed strong association as indicated by high boot strap value i.e., 92.7% at cluster node.

Cluster II: It embodied of six accessions (IGTGD-4, IGBANG-D-1, IGKMD-10, IG3108, IG 97-189 and IG 97-132) and shared minimum 77% genetic similarity. The intra-cluster similarity was ranging from 77 to100 %. Of these accessions, IG 97-189 and IG 97-132 shared 100% genetic similarity and occupied the same position in dendrogram. This cluster possessed accessions mostly dominated from southern part of India (IGTGD-4, IG BANG-D-1, 1GKMD-10 and IG3108) and two accession from northern part of India (IG97-189, and IG 97-132). Accessions from south India showed 83% genetic similarity among themselves, Which further joined to IG 3108 by 81% and 77% by IG97-189 and IG97-132. This cluster was separated from cluster I at 75% genetic similarity level.

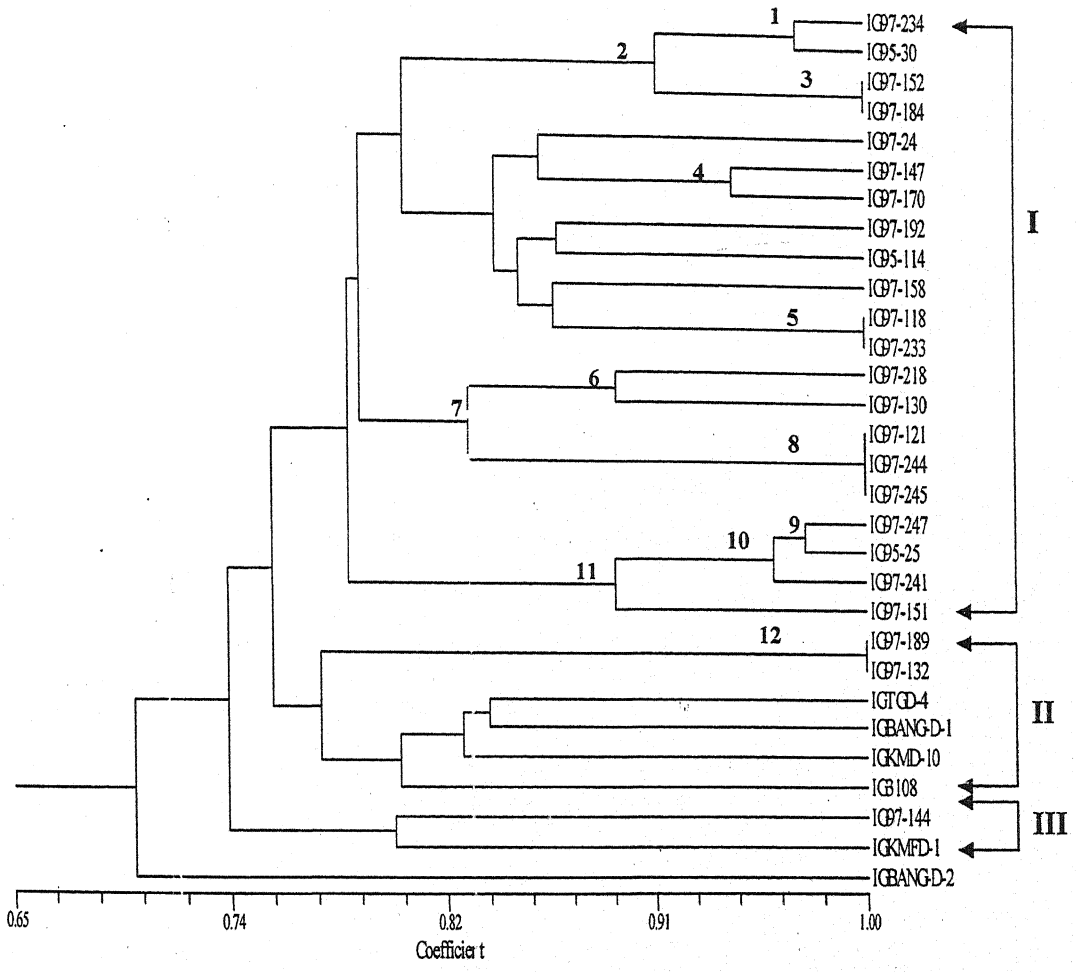


Fig. 7. Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on 106 STS bands.

Cluster III: It embodied only two accessions (IG 97-144 and IGKMFD-1). These two accessions grouped together at 80% similarity level, which was further separated from rest of cluster at 74% genetic similarity.

Boot strap analysis:

The reliability of dendrogram and clustering of accessions was evaluated with 1000 Boot straps using Win Boot software (Yap and Nelson, 1996). The boot strap value was ranged from 7.0 to 100% at various nodes of dendrogram which was a very wide range. Total 12 nodes in dendrogram were identified, which exhibited more than 50% bootstrap value. The bootstrap values of more than 50 % were indicated at major nodes in dendrogram .

Evaluation of ISSR primers for diversity estimate in 30 *Dichanthium* genotypes.

Five ISSR primers were used to construct dendrogram and establishment of genetic relationship among 30 *Dichanthium* genotypes. ISSR primers used in present study were highly rich with CA and GA sequence containing many repeats of [CA & GA] dinucleotide sequences. They were 10 –18 nucleotide long having GA / CA repeats. Among these primers, ISSR 80 and ISSR 81 were 10 nucleotides long with 4 repeats of CA dinucleotide sequence, where as primer ISSR 82, ISSR 83 and ISSR 84 were 18 nucleotides long with four repeat units of GACA sequence (Table 7).

A total of 61 amplified fragments were generated from five ISSR primers. The approximate molecular weight of these bands was ranged from 2000 to 200 base pair (Figure 8). An average of 12.2 bands per primer was recorded. Of these an average 10.4 bands per primer were observed to be polymorphic and 1.8 bands per primer were monomorphic. All 61 amplified fragments generated from these primers were prominent and distinguishable. Out of 61 bands scored 52 bands

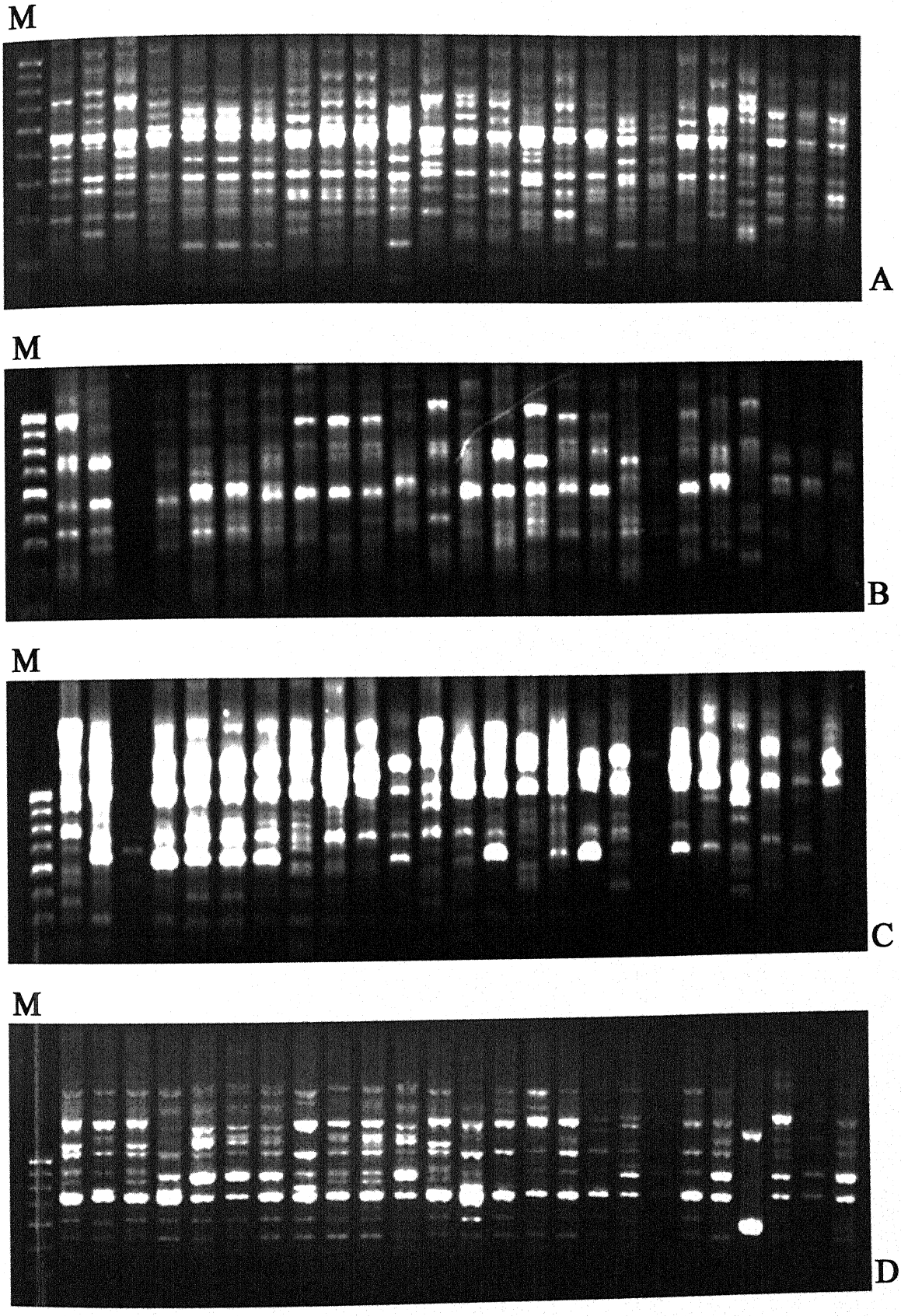


Fig. 8. ISSR profile with primers (A)ISSR-80, (B) ISSR-81, (C) ISSR-83, (D) ISSR- 84 in accessions of *Dichanthium*. M= 100 bp ladder

were polymorphic where as 9 bands were monomorphic. Polymorphism shown by these primers was ranged from 71.42 (ISSR 84) to 100% (ISSR 81). The average polymorphism i.e., 85.24% per primer was exhibited by these primers. Among these primers maximum (15) bands were generated from primer ISSR 80, Whereas 8, 12, 12, and 14 bands were scored from primers, ISSR 81, 82, 83 and ISSR 84 respectively (Table 7). It was observed that primers (ISSR 80 and ISSR 81) of 10 nucleotides sequence, with di-nucleotide repeat sequences were comparatively more polymorphic than those of having more than 10 nucleotides sequence long (ISSR 82, ISSR 83, and ISSR 84). High polymorphism i.e., 93.33 and 100 % was exhibited by primers ISSR 80 and ISSR 81 respectively. Three primers namely, ISSR 82, ISSR 83, and ISSR 84 were less polymorphic. The polymorphism exhibited by these primers was ranged from 83.33% (ISSR 82 and 83 each) to 71.42% (ISSR 84) polymorphism (Table 7). Among these ISSR primers, ISSR 84 produced total of 14 distinct bands of that 4 bands were monomorphic and 10 bands were polymorphic. Out of all the primers, the minimum (71.42%) polymorphism was detected in this (ISSR 84) primer. where as 2 primers namely ISSR82 and ISSR83 exhibited higher i.e., 83.33% polymorphism each. Two 10- mer ISSR primers namely ISSR80 and ISSR81 generated total 15 and 8 bands each respectively. Primer ISSR80 produced one monomorphic band and 14 polymorphic bands which ~~exhibited 93.33 % polymorphism.~~ already mentioned Primer ISSR 81 generated 8 polymorphic and ~~none of~~ no monomorphic bands, hence exhibited maximum (100%) polymorphism.

Polymorphic information content (PIC) and marker index (MI) obtained from the data of these primers were utilized in assessing the discriminatory and resolving power of these primer in genetic relationship study. The PIC value recorded among the primers was ranged from 0.405 to 0.500. The mean PIC i.e., 0.480 was obtained from these primers. Maximum PIC value was obtained from ISSR 84 (0.500), where as minimum i.e., 0.405 exhibited by ISSR 82. Two primers namely, ISSR 80 and ISSR 83 produced PIC i.e., 0.499 each, where as other two namely ISSR 81 and ISSR 84 exhibited maximum (0.5) PIC value each.

Table 7: ISSR primers used in 30 *Dichanthium* accessions .

Primer	Sequence 5'to 3'	Total bands	Polymorphic bands	%Polymorphic Bands	Monomorphic bands	PIC	Marker Index (MI)
80	GC[CA]4	15	14	93.33	01	0.499	6.99
81	GT[CA]4	08	08	100	-	0.500	4.00
82	[AGAC]4GC	12	10	83.33	02	0.405	4.05
83	AC[GACA]4	12	10	83.33	02	0.499	4.90
84	[GACA]4GT	14	10	71.42	04	0.500	5.00
Total		61	52		09		
Average		12.2	10.4	85.24	1.8	0.480	4.98

Primer ISSR82 indicated least (0.405) PIC value. Marker index (MI) values obtained from these primers were ranged from 4.00 to 6.99. The mean MI obtained was 4.98.The maximum MI (6.99) was reported in primer ISSR 80, where minimum (4.00) in ISSR81. Other primer namely, ISSR82 ISSR 83 and ISSR 84 exhibited MI values i.e., 4.05, 4.90 and 5.00 respectively. Higher PIC and MI value of ISSR marker indicated its usefulness detecting polymorphism among accessions estimating genetic relationship (Table 7).

Assessment of genetic diversity and clustering pattern of 30 *Dichanthium* accessions based on ISSR polymorphism data.

The estimation of genetic relationship and clustering pattern of 30 *Dichanthium* accessions was assessed using dendrograms generated from data obtained from five ISSR primers. Based on 61 amplified ISSR bands, a pair wise genetic distance based on Dice similarity coefficient was developed. Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using the Un-weighted pair group method with arithmetic mean (UPGMA) algorithm.

Dendrogram generated from ISSR data revealed 3 major clusters which together constituted 28 *Dichanthium* accessions (Figure 9). Two accessions namely IGBANG-D-2 and IG97-144 distinctly separated very early and joined rest of the accessions at 62.21 % and 56.77 % of genetic similarity respectively. Both IG BANG-D-2 and IG97-144 belongs to two different regions namely Bangalore (Karnataka) and Chattarpur (MP) of India. Rest of the 28 *Dichanthium* accessions clustered into three major clusters, represented as Cluster I, Cluster II and Cluster III. Genetic relationship among accessions belonging to 3 clusters was ranged from 66.6% to 100%. Where as genetic similarly among the clusters ranged from 66.6 % to 81.08 %.

Cluster I: Embodied four accessions namely IG97-234, IG95-30, IG97-152 and IG97-184, belonging to north India central plateau and exhibited minimum

81.08% genetic similarity. The Intra-cluster variability in similarity among the accessions of Cluster I was ranged from 81.08 to 100 %. Two accessions namely IG97-234 and IG95-30 shared 94% similarity and joined other two accessions namely IG97-152 and IG97-187 at 81.08 % similarity level. Further these two accessions (IG97-152 and IG97-187) exhibited 100% genetic similarity with each other. This cluster showed strong relationship among the accessions as indicated by high boot strap value (82.55) at this node.

Cluster II: It is the largest cluster, consisted of 17 accessions. Cluster II separated from cluster I at 66.6% genetic similarity and to Cluster III at 68.43% genetic similarity. Most of the accessions grouped in this cluster belonging to north India central plateau. Intermixing of one accession i.e., IGKMF-D-1 of south India in this cluster was also observed. Most of accessions clustered in this cluster shared more than 72% genetic similarity. Intra cluster variability in similarity ranged from 72.91% to 100%. Cluster II contained two sets of accessions. Each contained 2 accessions set one consisted of accessions namely IG 97-118 and IG 97 exhibited 100% genetic similarity with each other and other set contained two accessions namely, IG97-189 and IG 97-132 also shared 100% genetic similarity. Remaining 13 accessions of cluster II shared genetic similarity ranged from 72.91% to 96.10%. Cluster II It was further separated into two subclusters i.e., subcluster IIA and subcluster IIB. Subcluster IIA and IIB shared 72.91% genetic similarity.

Subcluster IIA: This cluster included 14 accessions and shared minimum 73.76% genetic similarity. Intra cluster variability in similarity was ranged from 73.76 to 100%. Within this subcluster (IIA), accession (IGKMFD-1) exhibited minimum (73.76 %) genetic similarity with rest of 13 accessions of the subcluster. Two accessions (IG97-118 and IG97-22) exhibited 100% genetic similarity. Whereas 11 accessions (IG97-24, IG97-192, IG97-218, IG 95-114, IG97-147, IG97-170, IG97-158, IG97-247, IG97-241, IG95-25, IG97-130) shared genetic similarity ranged from 77.95 to 96.10%. Among these accessions two accessions i.e., IG 97-192 and IG 97-218 were closely related and shared 89.2 % genetic

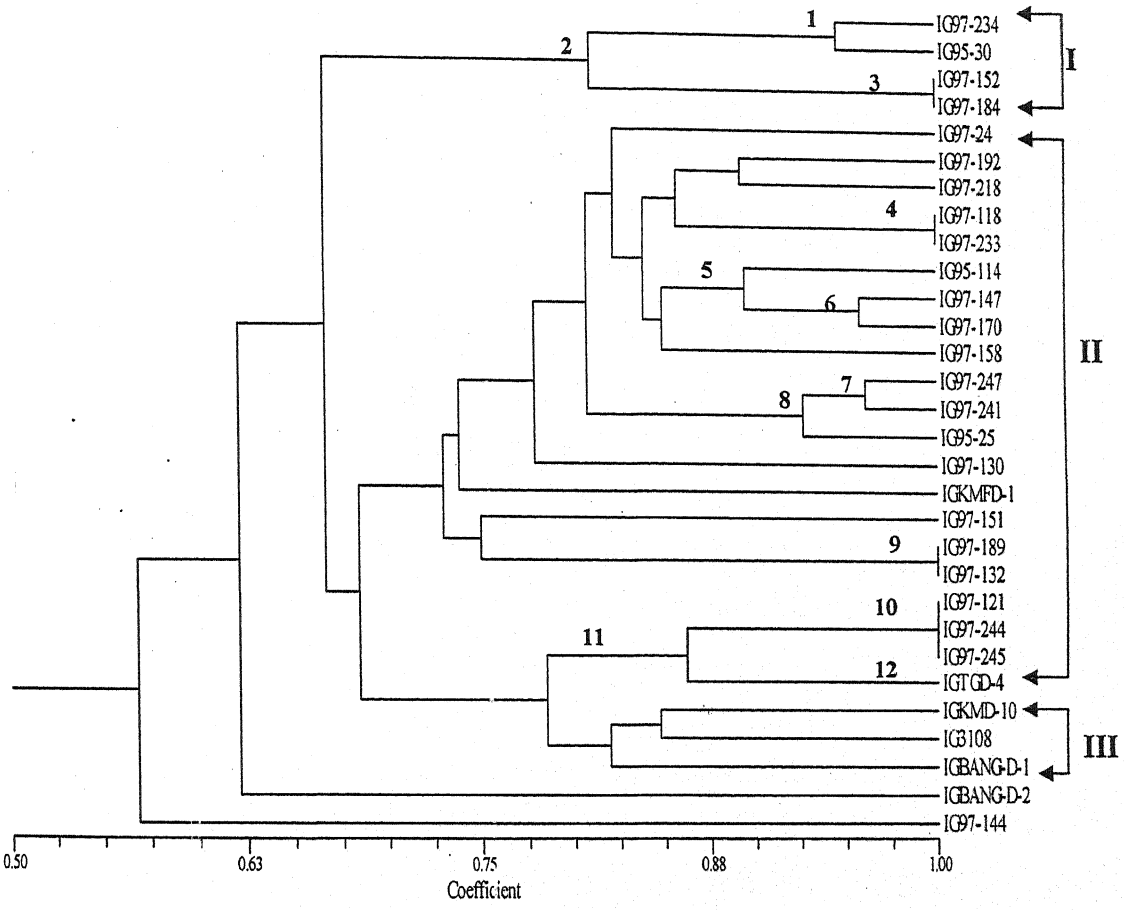


Fig. 9. Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on 61 ISSR bands.

similarity. A group of three accessions namely IG97-147, IG97-170 and IG 95-114 exhibited strong genetic association as the clearly indicated by boot strap values for this group i.e., 50.9. Two accessions namely IG97-147 and IG97-170 shared 95.7% similarity and also exhibited strong bootstrap value (79.3) at joining node. Further these two joined to IG 95-114 at 89.5 % genetic similarity this node also exhibited strong bootstrap value i.e., 50.9. These accessions further joined to accession IG 97-158 at 84.94% similarity.

Two accessions of subcluster IIA namely, IG97-247, IG 97-241 were also closely associated and shared 96.10 % genetic similarity, these accessions further joined to accession IG 95-25 and shared 92.63% similarity. Beside accession IGKMD-1, another accession IG97-130 showed minimum (77.95) genetic similarity with rest of the accessions of subcluster IIA.

Subcluster IIB: consisted of 3 accessions, these accessions of subcluster IIB shared 75% genetic similarity. Two accessions i.e., IG97-189 and IG97-132 shared 100% similarity and joined with the other accession (IG 97-151) of same subcluster at 75% similarity level.

Cluster III: It embodied seven accessions namely, IG97-121, IG97-244, IG97-245, IGTGD-4, IGKMD-10, IG3108 and IGBANG-D-1. This cluster was dominated by accessions belonging to south India. The intra cluster variability in similarity was ranged from 78.46 to 86.21%.A group of 3 accessions namely, IG97-121, IG97-244, IG97-245 belonging to north India occupied the same position in dendrogram and exhibited 100 % genetic similarity. These accessions were closer to one of the accession belonging to south India, i.e., IGTGD-4 joined at 86.21% similarity with high boot strap value i.e., (66.2) at joining node. Further Cluster III was separated from Cluster-II and Cluster I at 68.43% and 66.6% genetic similarity level respectively. All the accessions belonging to cluster III were grouped at minimum 78.46% genetic similarity level. A group of 3 accessions namelyIGKMD-10, IG3108 and IGBANG-D-1 belonging to south India clustered at 82.02% genetic similarity level. Two accessions namely IG

KMD-10 and IG 3108 exhibited 84.75% genetic similarity, which are further joined to accession IGBANG-D-1 at 82.02% similarity level.

Boot strap analysis:

The reliability of dendrogram was evaluated with 1000 Boot straps using Win Boot software (Yap and Nelson 1996). The boot strap value was ranged from 7.1 to 100% at various nodes of dendrogram which was a very wide range. Total 12 nodes in dendrogram were identified, which exhibited more than 50% boot strap value

Isozyme study:

Evaluation of four isozymes for diversity estimate in 30 *Dichanthium* genotypes:

The analysis of four isozymes viz., peroxidase (PRX, E.C. 1.11.1.17), esterase (EST, E.C. 3.1.1.2), polyphenol oxidase (PPO, E.C. 1.14.18.1) and superoxide dismutase (SOD, E.C. 1.15.1.1) was carried out using native polyacrylamide gel (10 %) electrophoresis method. All the four enzyme systems showed good resolution, staining and banding pattern was reproducible for each accession analyzed. The zymogram of gels was prepared by measuring the distance of each band from the point of separating gel and relative mobility (R_m) of each band was calculated as the ratio of distance traveled by the band to the tracking dye. Bands were numbered on the basis of increasing R_m values. Loci and alleles were subsequently numbered and lettered respectively. The isozyme patterns were defined by taking into account the number and position of bands. The banding pattern observed after polyacrylamide gel electrophoresis for these enzymes is presented for some accessions.

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Peroxidase (PRX):

In total 11 bands (isoforms) were generated with wide range of Rm value, it ranged from 0.072 to 0.50. These bands were given number from 1 to 11 in order to increasing Rm values (Table 8). These bands were together distributed into four distinct enzyme zones (loci) namely Locus 1 (PRX1), Locus 2 (PRX2), Locus 3 (PRX3) and Locus 4 (PRX4) consisted of 2, 2, 4 and 3 bands (alleles) (Figure 10). Out of 11 bands (isoforms) 4 were monomorphic and 7 polymorphic, which constituted 63.66% allelic polymorphism. Locus 3 (PRX3) was highly polymorphic (83.3%) and resulted maximum number of 4 alleles; locus 1 (PRX1) exhibited polymorphism in 16.6 % of accessions; locus 4 (PRX4) was monomorphic for all the accessions (table). Locus1 (PRX1) included 2 bands (isoforms) out of which one was polymorphic and one was monomorphic. Locus 2 (PRX2) included 2 bands and both were polymorphic. Locus 3 (PRX3) included 4 bands and all the bands were polymorphic, while Locus 4 (PRX4) included 3 bands and all the bands were monomorphic.(Table 8).The polymorphic information content (PIC) and marker index (MI) were 0.471 and 3.76 respectively (Table 8).

Esterase (EST):

A total of 22 bands (isoforms) were generated from this isozyme. The Rm value of bands ranged from 0.274 to 0.830.These bands were given number from 1-22 based on increasing their Rm value (Table 8). Out of 22 bands 20 bands were polymorphic whereas 2 monomorphic bands which accounts 90.905% polymorphism. Esterase produced four major zones of enzyme activity designated as locus EST1, EST2, EST3 and EST4; all these loci produced 7, 3, 5 and 7 alleles respectively (Figure 11). Out of 4 loci detected Locus 1 (EST1) and 4 (EST4) were highly polymorphic showing 80 and 96.6% of accessions respectively polymorphic, whereas Locus 2 (EST2) and Locus 3 (EST3) exhibited polymorphism i.e., 46.6 and 60.0% respectively. The polymorphic

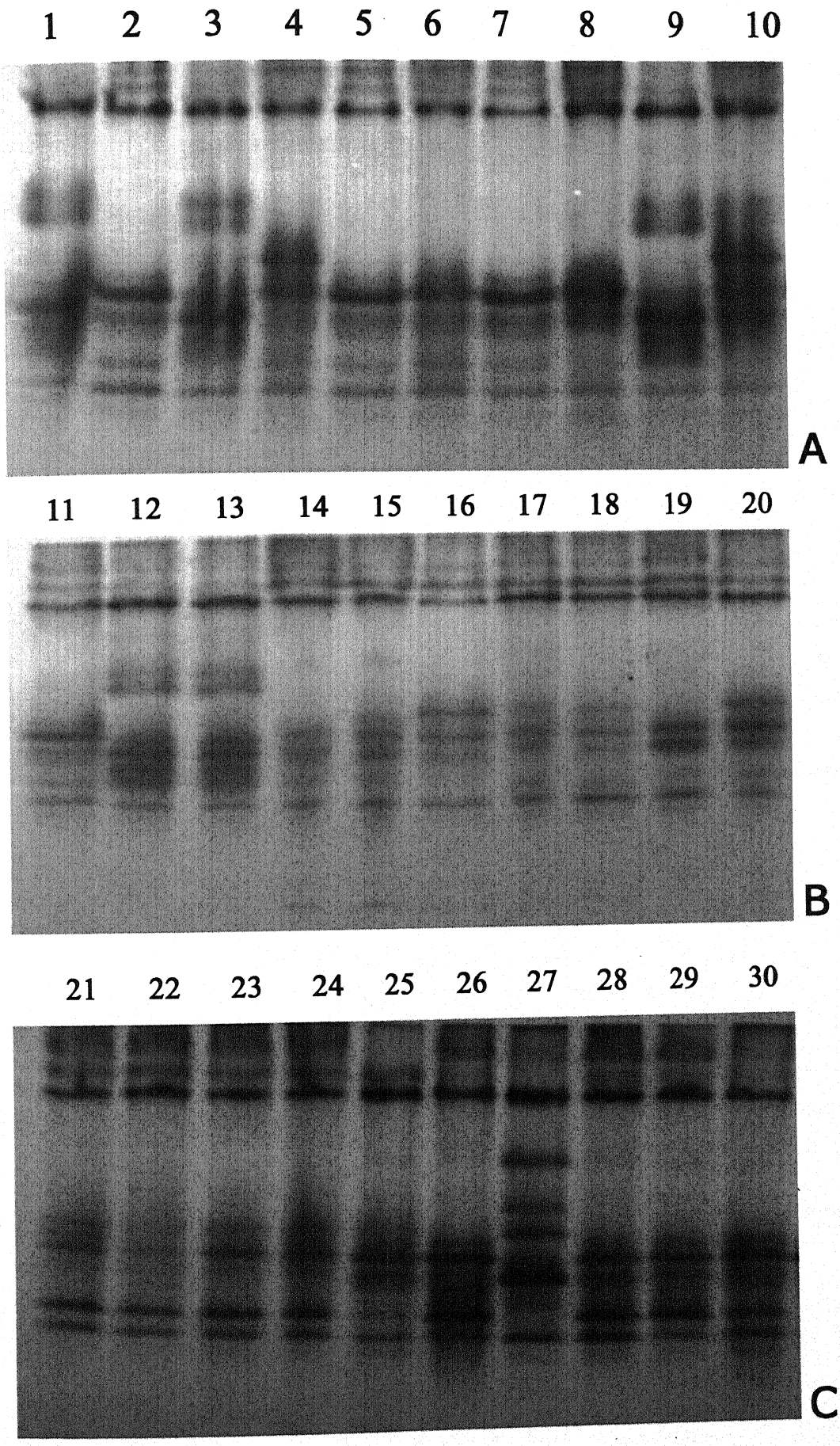

Fig.10. Isozyme profiles of peroxidase in control conditions for genetic diversity study in 30 accessions of *Dichanthium*

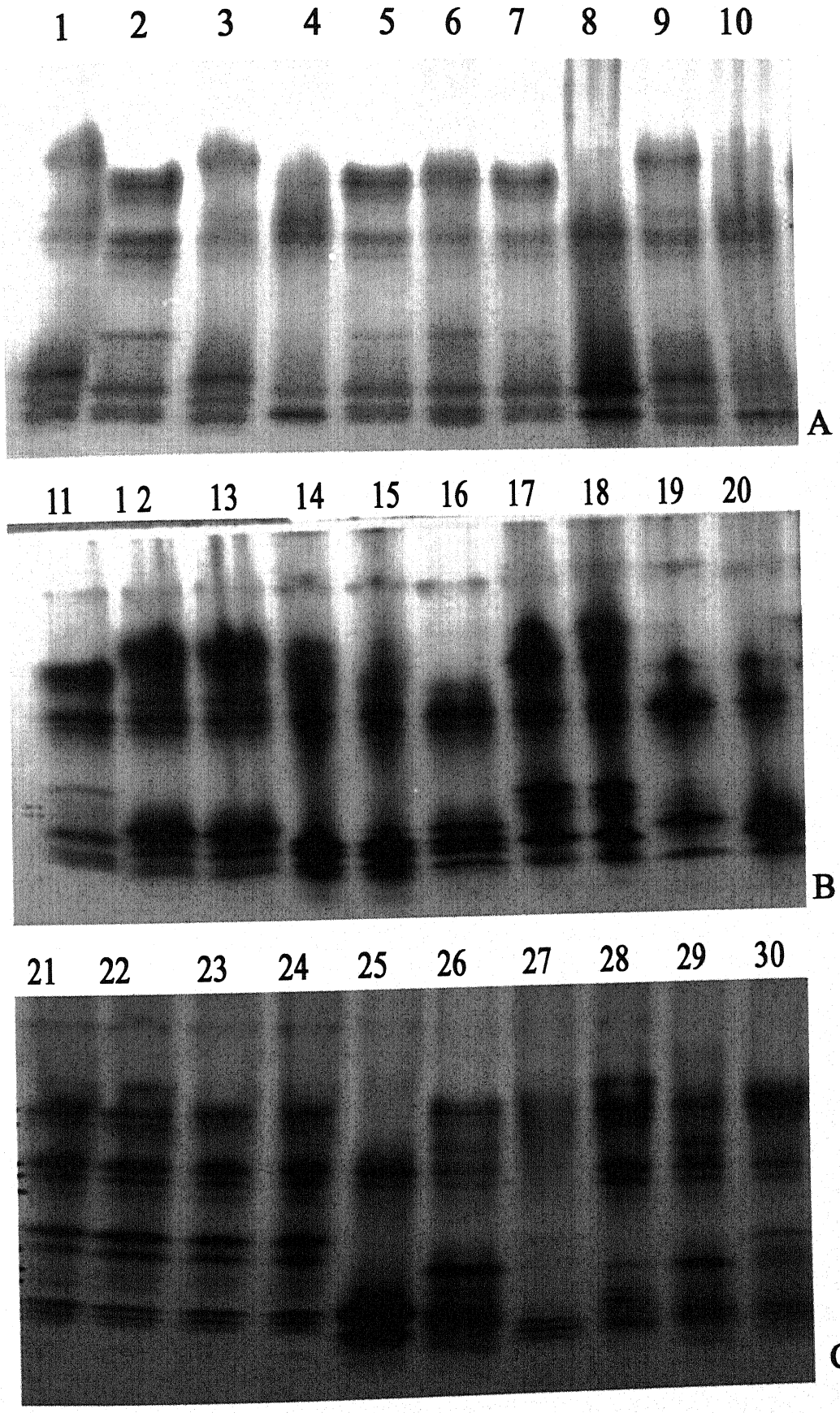

Fig.11. Isozyme profiles of esterase in control conditions for genetic diversity study in 30 accessions of *Dichanthium*

information content (PIC) i.e., 0.479 and marker index (MI) i.e., 9.58 were observed in esterase isozyme (Table 8).

Polyphenol oxidase (PPO):

It produced total 15 bands (isoforms) with Rm values ranged from 0.044 to 0.685. These bands were given number from 1 to 15 in order to increasing Rm values. Polyphenol oxidase produced maximum number of loci out of four isozymes studied. Five zones of enzyme activity were detected as PPO1, PPO2, PPO3, PPO4 and PPO5; these loci together generated 15 alleles (Figure 12). Out of 15 alleles 13 were polymorphic and 2 monomorphic. Total polymorphism exhibited by PPO was 86.66%. Locus 3 (PPO3) was maximum polymorphic where 90% of accessions were polymorphic and locus 2 was least where 23.33% accessions were polymorphic. The polymorphic information content (PIC) and marker index (MI) shown by PPO were 0.471 and 3.76 respectively (Table 8).

Superoxide dismutase (SOD) :

In total 7 bands (isoforms) were generated by SOD, with wide range of Rm value, it ranged from 0.405 to 0.920. These bands were given number from 1 to 7 in order to increasing Rm values. These bands were ~~together~~ distributed into three distinct enzyme zones (loci) designated as SOD1, SOD2 and SOD3 (Figure 13). Out of 7 bands (alleles), 3 bands were monomorphic and 4 bands were polymorphic, which exhibited 75% polymorphism enzyme wise. Out of three loci detected in SOD, Locus1 (SOD1) was monomorphic where as rest 2 loci i.e., SOD2 and SOD3 were polymorphic. Two alleles were observed at locus SOD1 and both were monomorphic for all thirty accessions. Locus SOD2 and SOD3 comprise 2 and 3 alleles respectively and were polymorphic. This enzyme appears to be least polymorphic (66.7%) of the four enzymes studied (Table). This enzyme showed low polymorphic information content (PIC) and marker index (MI), ~~there were~~ 0.299 and 1.196 respectively (Table 8).

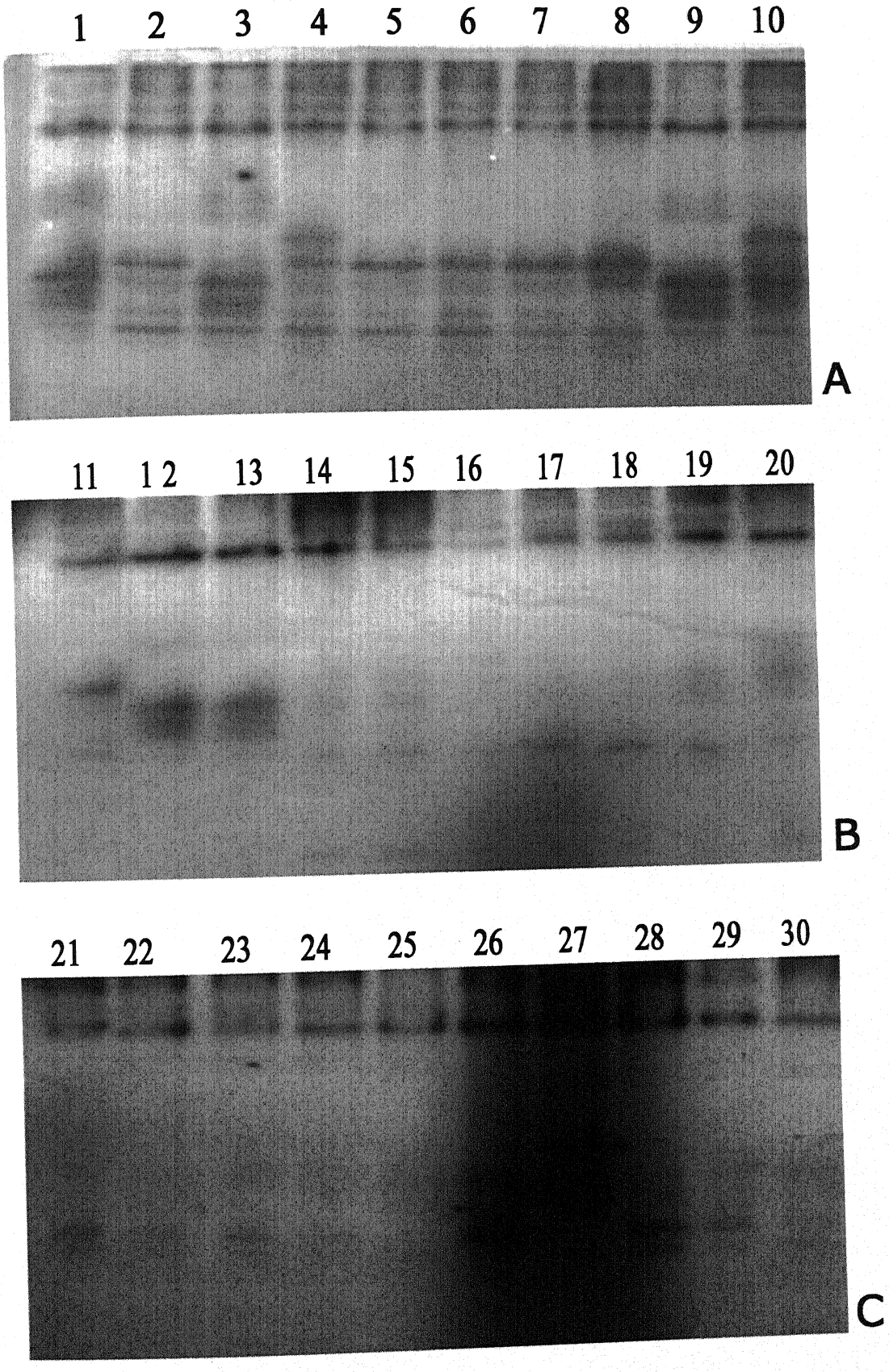

Fig.12. Isozyme profiles of polyphenol oxidase in control conditions for genetic diversity study in 30 accessions of *Dichanthium*

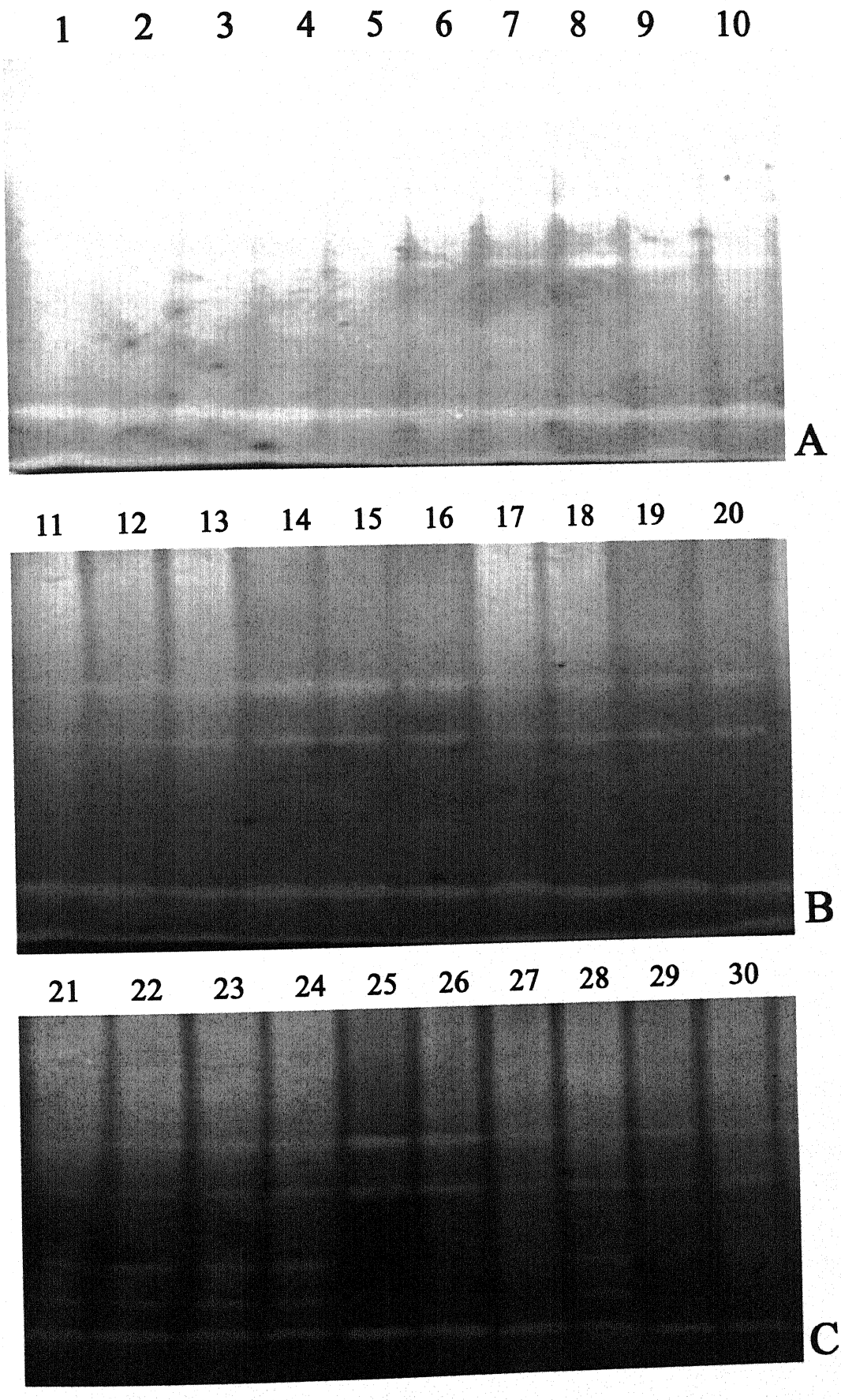

Fig.13. Isozyme profiles of superoxide dismutase in control conditions for genetic diversity study in 30 accessions of *Dichanthium*

Patterns of isozyme diversity:

All four enzyme systems showed variation in number of enzyme zones commonly known as locus. Maximum number of loci was detected in polyphenol oxidase and least in superoxide dismutase (Table 9). Four loci found in esterase and peroxidase each. Superoxide dismutase showed three loci designated as SOD1, SOD2 and SOD3 (Figure 14). Two alleles were observed at locus SOD1 and both were monomorphic for all thirty accessions. Locus SOD2 and SOD3 comprise 2 and 3 alleles respectively and were polymorphic (Table 9). This enzyme appears to be least polymorphic (66.7%) of the four enzymes studied. Esterase produced four major zones of enzyme activity designated as locus EST1, EST2, EST3 and EST4; all these loci produced 7, 3, 5 and 7 alleles respectively. Locus 1 and 4 were highly polymorphic showing 80 and 96.6% of accessions respectively polymorphic. Peroxidase enzyme observed four loci namely, PRX1, PRX2, PRX3 and PRX4 which generated a total of 11 alleles out of which 4 monomorphic and 7 were polymorphic. Locus 3 was highly polymorphic (83.3%) and resulting maximum number of 4 alleles; locus 1 exhibited polymorphism in 16.6 % of accessions; locus 4 was monomorphic for all the accessions investigated. Polyphenol oxidase produced maximum number of loci out of four isozymes studied. Five zones of enzyme activity were detected as PPO1, PPO2, PPO3, PPO4 and PPO5, these loci together generated 15 alleles. Out of 15 alleles 13 were polymorphic and 2 monomorphic. Locus 3 was maximum polymorphic where 90% of accessions were polymorphic and locus 2 was least where 23.33% accessions were polymorphic.

Out of the 14 polymorphic loci observed, EST4 showed polymorphic pattern in 96.6% of the accessions followed by PPO3 in 90% of the accessions. PRX1 was least polymorphic, where polymorphism was observed in only 16.6% of the accessions. This was followed by PPO2 with polymorphism in 23.33% of the total accessions. Though SOD2 and PRX2 loci were polymorphic each possessing two alleles were either present or absent in accessions and thus resulted no polymorphism at this locus. In case of SOD2 both alleles was present

Table 8 : Number and nature of loci and alleles as revealed by four isozymes in *Dichanthium annulatum*

Locus /allele	Esterase	Peroxidase	Polyphenol oxidase	Superoxide dismutase
Total no. of loci	4	4	5	3
No. of monomorphic loci	0	1	0	1
No. of polymorphic loci	4	3	5	2
Total no. of alleles	22	11	15	7
No. of monomorphic alleles	2	4	2	3
No. of polymorphic alleles	20	7	13	4
% polymorphism	90.9	63.36	86.66	57.14
Polymorphic information content (PIC)	0.479	0.471	0.498	0.229
Marker index (MI)	9.58	3.76	6.48	1.196

Table 9: Number of alleles at different locus and percentage of accessions polymorphic to the locus

Locus	No. of alleles detected	No. of polymorphic locus	Mean no. of alleles / polymorphic locus	% of accessions polymorphic to the locus
EST1	7	4	1.90	80.00
EST2	3	4	2.00	46.66
EST3	5	4	3.06	60.00
EST4	7	4	3.51	96.66
PRX1	2	3	1.00	16.66
PRX2	2	3	0.00	0.00
PRX3	4	3	2.60	83.33
PPO1	3	5	2.00	43.33
PPO2	3	5	1.43	23.33
PPO3	4	5	1.85	90.00
PPO4	3	5	1.00	46.66
PPO5	2	5	1.00	50.00
SOD2	2	2	0.00	0.00
SOD3	3	2	1.00	80.00

in five and absent in rest of 25 accessions. Similar situation was also observed with PRX2 but the accessions were different.

Polymorphic loci:

A total of 16 isozyme loci with 55 alleles were detected using four enzyme systems in 30 accessions of *Dichanthium*. The percentage of polymorphic loci ranged from 37.50 to 75(IG97-234) accession wise (Table 10). Accessions IG97-152, IG97-130, IG97-121, IG97-244, IG97-245 and IG97-144 depicted least (37.75%) polymorphism where 6 out of 16 loci were polymorphic. Maximum polymorphism (75%) observed in accession IG97-234 where 12 out of 16 loci were polymorphic. The number of polymorphic loci in other accessions ranged from 7 to 11 ~~out of 16 loci~~ with polymorphism ranging from 43.75 to 68.87. Out of 30 accessions 8 was detected possessing 50 % polymorphic loci where 8 out of 16 loci were polymorphic, the second highest (68.87%) polymorphic loci were observed in 4 accessions where 11 out of 16 loci were polymorphic followed by 6 accessions having 56.25 polymorphic loci. The number of accessions having 50% polymorphism was maximum (eight) followed by 43.75 and 37.75 each having six accessions. The number of monomorphic loci also varied among the accessions (Table 10). The maximum number of such loci was 10 observed in six accessions and minimum was 4 and observed in only one genotype IG97-234.

The allelic frequencies in 30 accessions of *Dichanthium* were calculated from 16 loci with 55 alleles (Table 11). The 16 enzyme coding loci coded by 4 enzyme systems, esterase was the most polymorphic enzyme system produced 22 alleles of which 20 was polymorphic. Locus 4 of esterase showing 7 alleles, 29 accessions out of 30 were polymorphic which accounts 96.6% of polymorphism to this locus. The second most polymorphic locus was PPO3 having 4 alleles polymorphic and 90% of accessions were polymorphic to this locus. PRX1 locus observed as the least polymorphic with only 16.6% of accessions were polymorphic out of 30 accessions, for rest of the polymorphic loci, 23.3 to 83.3 % of accessions were observed polymorphic (Table 11).

Table 10: Accessions wise description of the locus of different enzymes and percentage of polymorphic loci and heterozygosity in 30 accessions of *Dichanthium annulatum.*

Accession no.	EST (poly/ mono)	PRX (poly/ mono)	PPO (poly/ mono)	SOD (poly/ mono)	Total loci	Poly loci	Mono loci	% of poly loci acc. wise	Heterozygosity acc. wise
IG97-234	4/0	2/2	4/1	2/1	16	12	4	75.00	0.714
IG97-24	2/1	3/1	4/1	2/1	16	11	5	68.75	0.742
IG95-30	1/3	2/2	3/2	2/1	16	8	8	50.00	0.706
IG97-192	2/2	2/2	4/1	2/1	16	10	6	62.20	0.701
IG97-247	3/1	0/4	3/2	2/1	16	8	8	50.00	0.701
IG97-241	3/1	0/4	3/2	2/1	16	8	8	50.00	0.706
IG95-25	3/1	0/4	3/2	2/1	16	8	8	50.00	0.706
IG95-114	1/3	2/2	3/2	2/1	16	8	8	50.00	0.722
IG97-147	1/3	1/3	3/2	2/1	16	7	9	43.75	0.709
IG97-170	2/2	2/2	2/3	2/1	16	8	8	50.00	0.693
IG97-151	3/1	0/4	2/3	2/1	16	7	9	43.75	0.708
IG97-152	2/2	1/3	1/4	2/1	16	6	10	37.50	0.731
IG97-184	2/2	1/3	2/3	2/1	16	7	9	43.75	0.714
IG97-158	3/1	1/3	1/4	2/1	16	7	9	43.75	0.714
IG97-118	3/1	1/3	1/4	2/1	16	7	9	43.75	0.722
IG97-233	3/1	1/3	2/3	2/1	16	8	8	50.00	0.724
IG97-189	4/0	1/3	2/3	2/1	16	9	7	56.25	0.725
IG97-132	4/0	1/3	2/3	2/1	16	9	7	56.25	0.724
IG97-218	3/1	1/3	1/4	2/1	16	7	9	43.75	0.725
IG97-130	2/2	0/4	2/3	2/1	16	6	10	37.50	0.692
IG97-121	3/1	1/3	2/3	0/3	16	6	10	37.50	0.736
IG97-244	3/1	1/3	2/3	0/3	16	6	10	37.50	0.736
IG97-245	3/1	1/3	2/3	0/3	16	6	10	37.50	0.736
IG97-144	3/1	1/3	2/3	0/3	16	6	10	37.50	0.736
IGKMFD-1	4/0	1/3	3/2	2/1	16	10	6	62.50	0.726
IGTGD-4	3/1	1/3	4/1	2/1	16	10	6	62.50	0.724
IGBANG-D-2	4/0	2/2	3/2	2/1	16	11	5	68.75	0.739
IGKMD-10	4/0	1/3	3/2	0/3	16	8	8	50.00	0.743
IG3103	4/0	1/3	4/1	2/1	16	11	5	68.75	0.679
IGBANG-D-1	4/0	1/3	4/1	2/1	16	11	5	68.75	0.693

Table 11: Number of alleles, mean number of alleles per polymorphic locus, allele frequencies at polymorphic loci coding for four isozymes and heterozygosity of each locus.

Locus	Allele	Allele frequency	Heteroz-ygosity	No. of acc. poly. to locus	Mean no. alleles /poly locus	Locus	Allele	Allele frequency	Heterozy-gosity	No. of acc. poly. to locus	Mean no. of alleles poly locus
EST1	1	0.228	0.836	80.00	1.90	PRX3	1	0.212	0.721	83.33	2.6(
	2	0.211					2	0.317			
	3	0.105					3	0.342			
	4	0.087					4	0.129			
	5	0.087				PRX4	1	0.333	0.667	0.00	-
	6	0.175					2	0.333			
	7	0.105					3	0.333			
EST2	1	0.395	0.644	46.66	2.00	PPO1	1	0.221	0.649	43.33	2.0(
	2	0.395					2	0.389			
	3	0.210					3	0.389			
EST3	1	0.154	0.792	60.00	3.06	PPO2	1	0.307	0.663	23.33	1.42
	2	0.212					2	0.307			
	3	0.250					3	0.385			
	4	0.154				PPO3	1	0.435	0.662	90.00	1.8:
	5	0.230					2	0.177			
EST4	1	0.083	0.714	96.66	3.51		3	0.339			
	2	0.206					4	0.048			
	3	0.404				PPO4	1	0.532	0.607	46.66	1.0(
	4	0.037					2	0.234			
	5	0.140					3	0.234			
	6	0.196				PPO5	1	0.600	0.480	50.00	1.0(
	7	0.121					2	0.400			
PRX1	1	0.545	0.496	16.66	1.00	SOD1	1	0.500	0.500	0.00	-
	2	0.455					2	0.500			
PRX2	1	0.500	0.500	0.00	0.71	SOD2	1	0.500	0.500	0.00	0.7
	2	0.500					2	0.500			
						SOD3	1	0.750	0.406	80.00	1.0(
							2	0.125			
							3	0.125			

Genetic diversity parameters:

The mean number of alleles per polymorphic locus (Ap) as the measure of diversity calculated from 14 polymorphic loci out of total 16 loci detected presented in Table 5. Out of 14 polymorphic loci, EST2 and EST1 showed maximum value i.e., 3.51 and 3.05 mean alleles per locus, whereas the second highest number of Ap 2.6 was observed with locus PRX3. The least Ap value 1.0 was observed in loci PPO1, PPO4, PPO5 and SOD3. Rest of polymorphic loci showed Ap value in range of 1.3 to 2.0.

Mean heterozygosity observed among the polymorphic loci in four isozyme systems ranged from 0.406 to 0.836. Locus EST1 showed maximum 0.836 heterozygosity, the second highest mean heterozygosity values 0.792, 0.721 and 0.714 exhibited by locus EST3, PRX3 and EST4 respectively, whereas minimum 0.406 heterozygosity was shown by locus SOD3 (Table 11). At accession level the genetic diversity parameters like allele frequency, number of alleles and mean heterozygosity were calculated individually in 30 accessions studied (Table 10, 11). Mean heterozygosity ranged from 0.679 to 0.743 in different accessions. Maximum heterozygosity of 0.743 was observed in accession IGKMD-10. The accessions IGKMFD-1, IGTGD-4, IGBANGD-2 of south India had higher heterozygosity of greater than 0.70.

Assessment of genetic diversity and clustering pattern of 30 *Dichanthium* accessions based on four isozyme systems:

The similarity between the accessions was estimated using the Dice similarity matrix. Dendrogram based on SAHN clustering using UPGMA algorithm was generated for 30 *Dichanthium* accessions (Figure 15). The dendrogram based on 55 bands resulted from four enzyme systems (SOD, PPO, PRX and EST) of thirty accessions showed the presence of three major clusters designated as cluster-1, cluster- 2 and cluster-3.

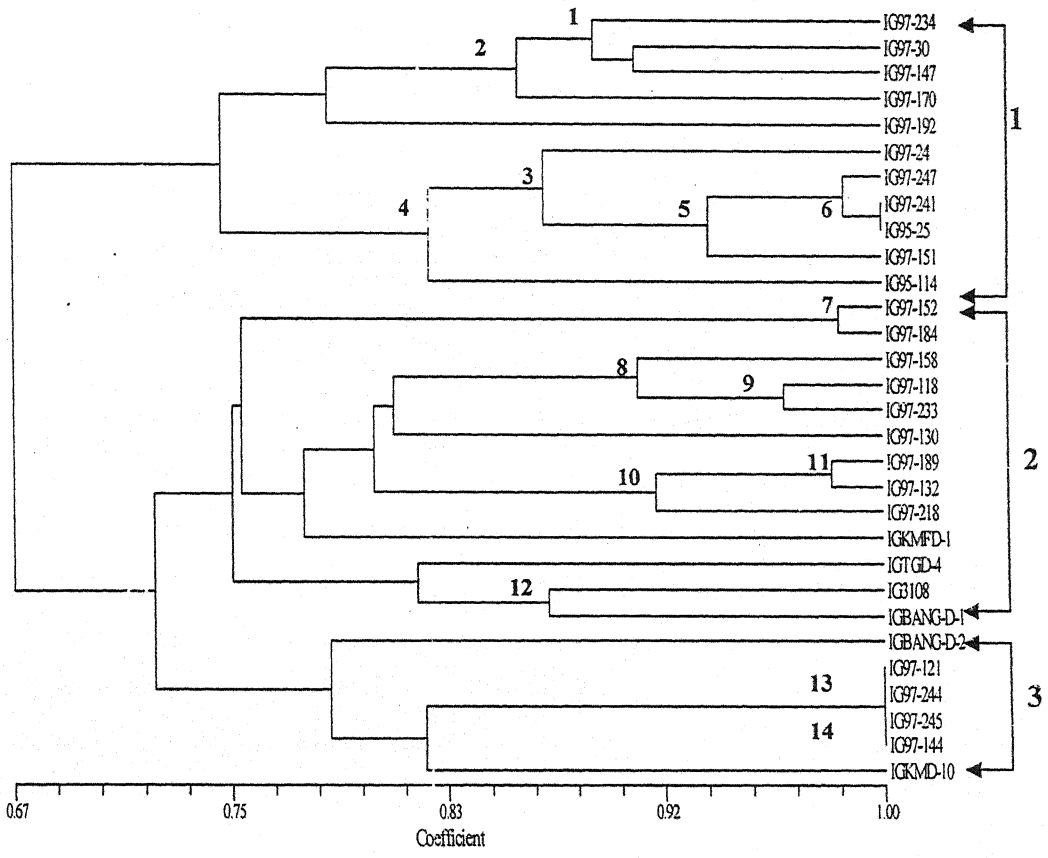

Fig. 15. Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on 55 isozyme bands.

Cluster-1: consisted of 11 accessions with genetic distance ranged from 0.00 to 0.253, cluster–2 consisted of 13 accessions with genetic distance ranged from 0.016 to 0.249 and finally cluster-3 consisted of 6 accessions with genetic distance ranged from 0.00 to 0.211. In cluster one IG97-241 and IG95-25 accessions were 100% similar and were collected from the close vicinity. This cluster was further subdivided into 2 sub-clusters. Cluster 1-1 comprised of 5 accessions of which four were collected from one region and one was from different region but these two regions were not too agro-ecologically diverse. The sub-cluster showed 79-90% similarity among them. Cluster 1-2 comprised of 6 accessions were largely collected from the same region as cluster 1-1. The members show 83-100% similarity. The sub-cluster 1-1 was 74.7% similar to sub-cluster 1-2.

Cluster 2: comprised of 13 accessions of diverse origin. In this cluster 100% similarity between any two accessions was not observed as the case with other clusters. The cluster was highly divergent as accessions of all the regions were represented. Of 13 accessions 9 was from central north India and rest 4 was from south India. The intra–cluster similarity ranged from 75 to 98%. It was further subdivided into three sub-clusters. Cluster 2-1 comprised of two accessions belonging to same region with 98% similarity. The sub-cluster was 76% similar to sub-cluster 2-2 which comprised of 8 accessions of central north India except one, IGKMFD-1, from south India. This accession, in fact, was in isolation and joined with sub-cluster 2-2 with 78% similarity. The similarity in intra-subcluster 2-2 ranged from 78 to 98%. Cluster 2-3 comprised of 3 accessions and all from south India, where IG3108 exhibited 87% similarity with IGBANG-D-1 and these two accessions showed 82% similarity with the third accession IGTGD-4.

Cluster 3: comprised of 6 accessions of which four accessions viz., IG97-121, IG97-244, IG97-245 and IG97-144 were 100% similar collected from central north India. These four subsets of accession joined with IGKMD-10 at 83% similarity level. The sixth accession of this cluster belongs to south India and showed 79% similarity with the five member of this cluster. Cluster 3 showed

72.2 similarity with cluster 2 and these two clusters together exhibited 67% similarity with cluster 1.

Boot strap analysis:

Total 14 nodes in dendrogram were identified, which exhibited more than 50% bootstrap value (Figure 15).

Stress studies

Water loss rate:

Leaves were cut into uniform size of 3.0 cm and weighed (fresh weight). They were left in petri dishes at room temperature and relative humidity 60-70% and allowed the evaporation of water from leaves. Each genotype was kept in triplicates. The leaves were weighed at interval of 2 hour, 4 hour and 24 hour. Loss in weight was calculated and subsequently the % loss in weight was determined. An average 44%, 56.17% and 65% weight loss was observed after 2hr, 4hr and 24hr respectively at room temperature. An average of 44.34± 1.82% weight loss was observed among 30 *Dichanthium* accessions after 2hr. It was ranged from 35.27± 1.37 (IG97-147) to 53.55± 4.86% (IG3108). Out of 30, seven accessions namely IG97-147,IG97-234,IG95-30,IG97-241,IG97-132,IG97-144 and IG97-121 reported least weight loss after 2 hr i.e., 35.27,36.02,37.35,38.15, 39.99,39.99 and 39.13% respectively. Weight loss in these accessions ranged from 35.27 to 39.99% with an average of 39.95%. Two accessions namely IG3108 and IG97-118 exhibited more i.e., 53.55% and 52.03% respectively water loss after 2 hr. Rest of 21 accessions exhibited loss in weight ranged from 40.77± 2.05 (IG95-25) to 49.64± 3.16 % (IGTGD-4) (Table 12).

After 4 hr, average weight loss ranged from 45.78± 4.78 to 71.68± 2.60 % with an average of 56.17% was recorded. Maximum weight loss (71.68%) was observed in IGTGD-4 where as minimum 45.78% in IG95-30. Among the 30 accessions a group of six accessions namely IGTGD-4, IG3108, IGKMD-10, IBANG-D-1, IG97-158 and IG97-158 exhibited more weight loss viz., 71.68, 66.76, 65.61, 61.04, 60.90 and 60.65% respectively after 4 hr at room temperature. It ranged from 60 to 75% with an average of 64.44%. A group of nine accessions showed less weight loss from 45 to 55%. These accessions were namely IG95-30, IG97-147, IG97-152, IG97-170, IG97-234, IG97-121, IG97-218, IG95-25, and IG97-241 exhibited less water loss viz. 45.78, 47.41, 49.19, 50.45, 50.58, 51.89, 53.97, 54.56 and 54.83% respectively. The average water loss of this group was 50.96%. Rest of the accessions exhibited moderate weight loss and that ranged from 55 to 59% (Table 12).

After 24hr nearly all accessions showed similar level of weight loss. It ranged from 60 to 75% with an average of 65.08%. Only three accessions namely IGTGD-4, IG3108 and IGKMD-10 exhibited more and they were 74.04, 73.15 and 70.56% respectively. Rest of the accessions showed 60 to 69% weight loss (Table 12).

Malondialdehyde (MDA) content:

The drought induced changes in the level of lipid peroxidation in term of malondialdehyde (MDA) content (nanomoles/gfw) was measured under both control and stress conditions. MDA content was significantly higher under water stress. Malondialdehyde content obtained in 30 *Dichanthium* accessions under well irrigated condition (control mean relative water content 90.10%) ranged from 92.51± 2.35 to 200.0± 6.92 nanomoles/gfw with an average of 130.88±3.33 nmole/gfw. Maximum MDA content (200.0± 6.92 nanomoles/gfw) was observed in IG97-144 and minimum 92.51± 2.35 nanomoles/gfw in IG97-130 accessions. MDA content under stress condition (at mean RWC 49.95%) increased in all the accessions (Table 13).

Table 12: Water loss rate in different accessions of *Dichanthium*.

Accession	%Wt loss (2hr)	%Wt loss (4hr)	%Wt loss (24hr)
IG97-234	36.02±2.731	50.58±5.634	60.44±0.854
IG97-24	41.57±1.053	57.45±5.548	67.86±1.486
IG95-30	37.35±1.026	45.78±4.789	59.18±0.666
IG97-192	47.73±1.937	58.99±4.096	67.57±1.135
IG97-247	49.51±1.440	60.65±2.400	66.90±1.312
IG97-241	38.15±1.345	54.83±6.035	65.44±1.452
IG95-25	40.77±2.055	54.56±2.164	62.75±0.563
IG95-114	44.05±0.670	55.58±1.260	64.63±0.494
IG97-147	35.27±1.372	47.41±2.841	60.66±1.164
IG97-170	41.38±0.995	50.45±6.377	62.81±2.077
IG97-151	48.12±0.810	58.44±2.688	60.07±8.116
IG97-152	42.9±3.830	49.19±6.859	60.04±1.489
IG97-184	45.59±2.457	54.14±2.638	61.28±1.027
IG97-158	48.87±0.761	60.90±3.238	66.43±2.213
IG97-118	52.03±2.862	58.03±2.410	67.12±7.142
IG97-233	48.76±0.796	59.17±3.225	64.39±4.331
IG97-189	41.02±2.118	57.28±2.879	66.43±1.699
IG97-132	39.99±2.630	55.19±4.910	63.83±1.047
IG97-218	43.26±1.363	53.97±3.275	61.43±0.832
IG97-130	46.88±1.896	57.64±2.774	60.63±0.288
IG97-121	39.13±1.747	51.89±0.664	65.08±2.642
IG97-244	46.98±1.118	56.17±6.150	69.42±0.780
IG97-245	45.14±4.323	58.67±3.120	65.78±1.377
IG97-144	39.99±1.654	59.93±4.485	69.55±1.319
IGKMFD-1	44.69±1.575	57.61±2.067	67.80±2.108
IGTGD-4	49.64±3.164	71.68±2.660	74.04±1.431
IGBANG-D-2	48.38±0.441	56.85±0.730	59.64±1.143
IGKMD-10	48.48±0.892	65.61±4.536	70.56±2.129
IG3108	53.55±0.860	66.76±5.028	73.15±3.344
IGBANG-D-1	44.91±0.903	61.04±4.998	67.67±1.302
Mean	**44.34±1.828**	**56.17±4.851**	**65.089 ±1.899**

Malondialdehyde (MDA) content under water stress condition ranged from 92.74± 4.00 (IG97-130) to 210.05± 2.53 nanomoles/gfw (IG97-144) with an average of 160.12 ± 3.61 nanomoles/gfw. The average 23.22% increase was recorded in *Dichanthium* accession under stress. Maximum increase in MDA content i.e., 68.06% (form 110.15 to 185.13 nanomoles/gfw control to stress) was observed in IGKMFD-1 over its control where as minimum 0.25% (from 92.51 to 92.74 nanomoles/gfw) in IG97-130. Nine accessions namely IGKMFD-1, IG97-152, IG97-241, IG97-158, IG95-114, IG97-24, IG95-25, IG95-30, and IG97-121 of the 30 the accessions showed major increase in MDA content i,e. 68.06, 55.16, 50.8, 40.041, 47.25, 40.47, 39.40, 34.91 and 33.76% respectively at high magnitude of drought stress. It ranged from 30 to 68 % over their respective control with an average increase of 44.88%. Out of 30, seven accessions namely IG97-151, IG97-118, IG97-233, IG97-132, IG97-130, IG97-144 and IGKMD-10 did not show significant difference in MDA increase form their respective control. Fourteen accessions namely IG97-234, IG97-192, IG97-247, IG97-147, IG97-170, IG97-184, IG97-189, IG97-218, IG97-244, IG97-245, IGTGD-4, IGBANG-D-2, IG3108 and IGBANG-D-1 showed moderate increase in MDA content that ranged from 10.7 to 29.8% increase in MDA content from their controls with an average of 17.3% (Table 13).

Proline content accumulation:

Proline was estimated using leaf tissues from both control and stressed plants of *Dichanthium* accessions. Proline level was measured in control plants of 30 *Dichanthium* accessions where RWC ranged from 82.82 ± to 97.07% with an average of 91.87%. Variation of proline level at control condition was observed among *Dichanthium* accessions and ranged from 1.15 ± 0.031 to 8.10 ± 0.270 µmoles/gram fresh weight with an average of 2.89µmoles/gfw. Higher level of proline was observed in accessions namely IG97-151, IG97-212, and IG97-24 i.e., 8.10± 0.126, 7.17± 0.328 and 5.53± 0.318 µmoles/gram fresh weight

Table 13: Malondialdehyde (MDA) content (nanomoles gfw^{-1}) in *Dichanthium* accessions under control and stressed condition

Accession	Nanomoles/ gfw (control)	Nanomoles/gfw (Stress)	RWC (%) control	RWC (%) stress	Increase (%) C to S
IG97-234	115.74±10.21	134.91±2.48	85.32±1.92	57.95±0.07	16.56
IG97-24	138.34±1.10	194.33±5.16	90.34±0.01	52.53±0.65	40.47
IG95-30	139.70±0.58	188.45±0.52	95.05±0.05	47.40±1.97	34.90
IG97-192	135.81±1.19	163.23±2.34	90.27±1.88	46.70±2.40	20.18
IG97-247	154.41±6.14	185.78±3.19	91.39±3.50	48.90±1.27	20.31
IG97-241	134.49±1.63	202.83±10.2	83.15±4.94	50.10±2.12	50.81
IG95-25	116.34±3.81	162.18±4.85	90.01±2.36	47.06±3.19	39.40
IG95-114	101.95±5.70	150.12±2.26	86.16±3.34	53.76±2.02	47.25
IG97-147	127.79±2.26	157.92±2.47	92.86±1.63	48.66±0.94	23.58
IG97-170	121.57±0.95	150.52±1.75	91.06±0.96	52.17±1.63	23.81
IG97-151	139.49±1.24	142.37±4.83	84.49±1.57	51.41±1.29	2.067
IG97-152	104.63±3.91	162.36±2.93	89.94±0.09	51.00±2.82	55.16
IG97-184	132.93±4.17	159.54±2.51	94.98±2.45	61.10±0.14	20.01
IG97-158	109.04±0.94	152.71±2.85	85.64±0.01	57.55±1.20	40.04
IG97-118	98.76±7.58	105.07±4.50	84.20±3.54	55.17±0.24	6.389
IG97-233	107.12±5.61	114.28±4.44	88.20±2.03	57.44±0.79	6.690
IG97-189	149.54±2.60	165.65±3.59	96.35±1.02	37.48±1.24	10.77
IG97-132	153.97±3.44	160.71±2.04	91.01±0.65	41.76±0.33	4.377
IG97-218	106.09±3.06	121.98±3.01	89.11±1.11	44.77±0.31	14.97
IG97-130	92.51±2.35	92.74±4.00	96.18±0.96	52.65±1.20	0.252
IG97-121	156.17±3.16	208.89±1.36	88.37±2.82	46.92±1.52	33.76
IG97-244	134.09±4.41	152.06±4.53	88.22±0.99	48.79±2.53	13.40
IG97-245	135.37±2.00	156.40±5.30	88.51±0.48	55.84±0.21	15.53
IG97-144	200.45±6.29	210.05±2.53	92.64±2.54	58.32±3.28	4.789
IGKMFD-1	110.15±6.84	185.13±3.90	89.40±1.00	55.16±0.23	68.06
IGTGD-4	123.77±1.09	146.10±4.47	90.18±1.07	41.25±1.76	18.03
IGBANG-D-2	129.07±0.65	156.53±3.67	93.44±0.56	40.59±0.83	21.27
IGKMD-10	164.87±3.36	169.91±4.39	91.80±0.60	46.30±1.83	3.058
IG3108	140.26±1.31	182.16±5.58	95.62±1.47	46.50±2.12	29.86
IGBANG-D-1	152.00±2.464	168.77±2.68	89.40±1.80	43.25±2.47	11.03
Mean	**130.88±3.34**	**160.12±3.61**	**90.11± 2.42**	**49.95± 3.66**	**23.22**

* LSD at (P<0.05) 8.25

** LSD at (P<0.01) 10

respectively. Proline content measured in other accessions ranged from 1.20± 0.045 (IG97-192) to 4.65 ± 0.042 (IGBANG-D-1). A significant increase in proline level was observed under water stress (mean RWC 49.1%) condition among the accessions. It increased 25 -163 fold among accessions from their respective control values. Out of 30 accessions, 16 exhibited more than 60 fold increase [60.4 (IG97-234) to 163.8 (IGBANG-D-2)] over their control, whereas in rest of the accessions it was 25.21 (IG97-170) to 58.62 (IG97-132) fold increased. Under water stress condition, proline level observed in the range of 11.12 (IG97-233) to 360 (IGBANG-D-2) µmoles/gram fresh weight with an average of 173.0 µmoles/ gram fresh weight. So, under stress average proline content increased from 2.89 (control) to 173 (stress) µmoles/gram which amounts to an average 70.92 fold increase among the accessions. Maximum proline content (360 µmoles/gram fresh weight) was observed in IGBANG-D-2 and second highest (333.75 µmoles/gram fresh weight) in IG97-24 and IG97-151 each. Minimum proline content (11.12 µmoles/gram fresh weight) was observed in IG97-233. A group of six accessions namely IGBANG-D-2, IG97-118, IG97-147, IGKMFD-1, IGKMD-10 and IG97-130 showed more than 100 fold increase in proline content over their control (i.e., 163.3, 162.8,156,149.3,126.9 and 108.6 fold respectively), whereas 3 accessions namely IG97-170, IG97-241, and IG97-121 showed minimum i.e., less then 30 fold increase in their proline content (i.e., 25.21, 29.01 and 29.28 fold respectively) (Table 14).

Total soluble protein:

Change in total soluble protein was measured in 30 *Dichanthium* accessions under stressed condition. Protein content in non stressed (control) accessions was ranged from 15.71 ± 3.09 to 51.99 ± 0.634 mg/gfw with an average of 33.06 ± 1.25mg/gfw. Accession IG97-132 showed maximum protein content i.e., 51.99 ± 0.634 mg/gfw and minimum 15.71 ± 3.09 mg/gfw in IG97-192. Protein content under water stress increased uniformly in all the accessions. A significant difference was observed in protein content under water stress condition in all the

Table 14: Change in proline level in water stressed plants of 30 *Dichanthium* accessions

Accession	µmole gfw^{-1} (control)	µmole gfw^{-1} (stress)	RWC (%) control	RWC (%) stress	Fold changein proline Cto S
IG97-234	1.69±0.20	102.2±2.86	90.60 ± 3.05	47.0 ±1.25	57.7
IG97-24	5.35±0.31	333.75±5.71	89.28± 0.91	62.58± 0.30	61.2
IG95-30	1.525±0.11	143.16±1.56	92.21± 0.10	45.04± 0.04	92.9
IG97-192	1.20±0.04	42.25±0.54	91.32± 1.09	48.02± 2.60	34.2
IG97-247	2.35±0.11	89.50±5.60	89.72± 2.45	53.58± 2.53	36.9
IG97-241	2.55±0.21	74.0±4.19	95.45± 0.07	47.57± 0.15	27.84
IG95-25	1.65±0.11	124.25±1.76	92.25± 0.40	50.91± 1.02	74.3
IG95-114	1.45±0.1	227.0±6.41	92.80± 1.59	48.31± 1.36	155.5
IG97-147	2.85±0.07	208.25±2.72	92.01± 0.05	41.55± 2.29	72.0
IG97-170	1.15±0.02	29.0±2.03	92.83± 0.89	58.11± 0.68	24.2
IG97-151	8.10±0.21	333.75±6.37	93.58± 0.18	44.97± 0.41	40.1
IG97-152	4.07±0.27	283.0±9.75	89.27± 0.72	43.21± 1.21	68.5
IG97-184	4.10±0.25	265.0±6.57	90.0 ± 1.12	43.5.0± 1.70	63.6
IG97-158	4.32±0.12	245.0±6.31	82.82± 0.14	44.45± 0.99	55.7
IG97-118	1.30±0.02	211.75±5.03	92.39± 1.38	53.28± 0.14	161.8
IG97-233	1.27±0.08	11.12±7.51	92.10± 2.12	65.81± 1.41	86.5
IG97-189	2.27±0.01	120.24±4.59	90.00± 0.05	48.06± 0.94	51.9
IG97-132	3.97±0.08	232.75±2.65	97.07± 1.61	47.68± 0.83	57.6
IG97-218	2.60±0.09	211.5±3.93	95.25± 2.03	46.19± 1.39	80.3
IG97-130	2.97±0.13	322.75±8.95	91.25± 1.66	43.29± 0.18	107.6
IG97-121	7.17±0.32	21.05±3.60	92.46± 0.90	48.64± 0.82	28.3
IG97-244	3.35±0.09	102.5±2.14	96.69± 1.84	61.66± 1.15	29.5
IG97-245	3.35±0.14	170.75±9.40	93.98± 0.47	60.66± 0.08	49.9
IG97-144	3.67±0.12	212.25±2.86	93.09± 1.85	53.47± 1.13	56.8
IGKMFD-1	1.45±0.13	216.50±1.06	91.83± 0.16	47.25± 1.58	148.3
IGTGD-4	1.15±0.03	46.0±2.54	89.05± 0.28	55.36± 0.88	39.1
IGBANG-D-2	2.20±0.03	360.0±5.43	92.48± 2.86	46.04± 0.91	162.
IGKMD-10	1.65±0.09	109.4±2.20	92.11± 0.03	46.78± 0.75	65.3
IG3108	2.07±0.04	80.0±3.08	90.12± 1.35	49.10± 2.28	37.6
IGBANG-D-1	4.65±0.04	235.0±2.41	92.15± 0.31	49.44± 2.87	49.5
Mean	**2.91± 0.04**	**181.70± 3.42**	**91.87±0.034**	**49.38±2.87**	**61.30**

* LSD at (P<0.05) 19.37

**LSD at (P<0.01) 25.54

accessions. The protein content obtained under stress condition among *Dichanthium* accessions ranged from 19.91± 0.252 to 81.22 ± 0.580 mg /gfw, with an average of 47.99 ± 1.77 mg/gfw. The maximum protein was observed in IGBANG-D-1 (i.e., 81.22 ± 0.580 mg/gfw) and minimum in IG97-192 (i.e., 19.91± 0.252 to 81.22 ± 0.580 mg /gfw). Under water stress an average 45.21% increase in protein content was recorded among the accessions. It ranged from 4.48 (IG97-170) to 117.83 % (IG3108) from their respective controls with an average of 45.21. Out of 30, seven accessions namely IG3108, IG97-121, IGBANG-D-1, IG97-192, IG95-30, IG97-234, and IG97-151 exhibited maximum increase of protein content (117.3, 113.27, 106.62, 101.8, 98.60, 77.84 and 68.01 % respectively) with an average of 97.63 %. Fifteen accession exhibited moderate increase in protein content [1.26 (IG97-118) to 66.31% (IGKMF-D-1)] with an average of 43.59 %, whereas rest of 7 accessions showed least increase in protein (4.48 to 17.72%) with average increase of 15.60 % from their respective control (Table 15).

Total osmolyte accumulation :

Total osmolyte concentration (osmolality) was measured in control plants of 30 *Dichanthium* accessions where RWC level ranged from 82.82 ± to 97.07%. Osmolality among the control plants in *Dichanthium* accessions ranged from 404.3 ± 2.05 to 752 ± 2.3 mmoles/kg with an average of 570.47± 15.22 milimoles/kg. Maximum osmolality 752.0 milimoles/kg observed in IG97-24 and minimum 404.3 milimoles/kg in IGTGD-4. A pair wise't' test for significance difference in osmolality between control and stressed plants of individual accession indicated significance difference in all accessions. Osmolality observed in water stressed plants at an average 49.38 % RWC, osmolality ranged from 840±3.26 milimoles/kg to 1594±3.26 milimoles/kg with an average value of 1243.8± 15.2 milimoles/kg. Maximum osmolality 1594± 3.26 milimoles/kg observed in IG94-24 and minimum 840± 3.26 milimoles/kg in IG97-244. Percent wise increase in osmolality was ranged from 26.9 to 247.7% among the

Table 15: Change in total soluble protein under water stressed plants.

Accession	Control mg/gfw	Stress mg/gfw	RWC (%) control	RWC (%) stress	% increase
IG97-234	21.37 ±0.88	38.01 ±2.12	90.60 ± 3.05	47.0 ±1.25	77.8412
IG97-24	19.82 ±0.38	22.68 ±1.33	89.28 ± 0.91	52.58 ± 0.30	14.40678
IG95-30	21.71 ±2.59	43.11 ±2.62	92.21 ± 0.10	45.04 ± 0.04	98.60868
IG97-192	17.89 ±0.66	36.12 ±0.63	91.32 ± 1.09	48.02 ± 2.60	101.8779
IG97-247	15.71 ±3.07	19.91 ±0.25	89.72 ± 2.45	53.58 ± 2.53	26.73797
IG97-241	18.98 ±2.57	27.80 ±1.14	95.45 ± 0.07	47.57 ± 0.15	46.46018
IG95-25	43.68 ±0.76	50.65 ±1.76	92.25 ± 0.40	50.91 ± 1.02	15.96154
IG95-114	22.51 ±0.14	33.35 ±0.88	92.80 ± 1.59	48.31 ± 1.36	48.13433
IG97-147	35.87 ±2.38	54.18 ±0.75	92.01 ± 0.05	41.55 ± 2.29	51.05386
IG97-170	41.16 ±0.14	43.01 ±0.52	92.83 ± 0.89	58.11 ± 0.68	4.489796
IG97-151	20.75 ±0.14	34.86 ±1.89	93.58 ± 0.18	44.97 ± 0.41	68.01619
IG97-152	30.99 ±0.66	46.20 ±0.38	89.27 ± 0.72	43.21 ± 1.21	49.05149
IG97-184	33.89 ±1.43	51.94 ±0.85	90.0 ± 1.12	43.5.0± 1.70	53.24875
IG97-158	48.80 ±0.29	57.46 ±2.30	82.82 ± 0.14	44.45 ± 0.99	17.72806
IG97-118	47.80 ±0.76	57.96 ±5.05	92.39 ± 1.38	53.28 ± 0.14	21.26538
IG97-233	34.77 ±1.01	53.17 ±1.26	92.10 ± 2.12	55.81 ± 1.41	52.89855
IG97-189	34.66 ±0.38	53.26 ±3.25	90.00 ± 0.05	48.06 ± 0.94	52.77108
IG97-132	51.99 ±0.63	70.14 ±0.58	97.07 ± 1.61	47.68 ± 0.83	34.89499
IG97-218	44.18 ±0.88	49.64 ±1.09	95.25 ± 2.03	46.19 ± 1.39	12.35741
IG97-130	43.09 ±1.40	54.26 ±2.39	91.25 ± 1.66	43.29 ± 0.18	25.92593
IG97-121	30.99 ±1.51	66.11 ±4.42	92.46 ± 0.90	48.64 ± 0.82	113.2791
IG97-244	38.89 ±3.62	45.78 ±2.71	96.69 ± 1.84	51.66 ± 1.15	17.71058
IG97-245	38.81 ±2.56	52.75 ±2.81	93.98 ± 0.47	50.66 ± 0.08	35.93074
IG97-144	39.90 ±2.39	66.36 ±1.82	93.09 ± 1.85	53.47 ± 1.13	66.31579
IGKMFD-1	39.98 ±0.88	49.31 ±1.47	91.83 ± 0.16	47.25 ± 1.58	23.31933
IGTGD-4	21.84 ±0.52	17.30 ±0.38	89.05 ± 0.28	55.36 ± 0.88	66.07143
IGBANG-D-2	33.68 ±1.02	39.65 ±0.14	92.48 ± 2.86	46.04 ± 0.91	17.70574
IGKMD-10	21.51 ±0.88	23.52 ±0.38	92.11 ± 0.03	46.78 ± 0.75	8.949416
IG3108	37.21 ±2.02	81.06 ±2.11	90.12 ± 1.35	49.10 ± 2.28	117.833
IGBANG-D-1	39.31 ±0.91	81.22 ±0.58	92.15 ± 0.31	49.44 ± 2.87	106.6239
Mean	**33.06 ±1.25**	**47.36 ±1.77**	**91.87 ± 0.34**	**49.38 ±2.87**	**45.12577**

* LSD at (P<0.05) 4.045
**LSD at (P<0.01) 5.340

accessions under stress. The mean increase of 123.20% osmolality was observed among the accessions under stress. A maximum 247.7% increase was observed in IGBANG-D-2 where as minimum (26.69%) increase in IG97-244. Out of 30 accessions, 21 accessions increased their osmolality more than 100 % over their control under water stress where it ranged from 103.29 (IG97-247) to 247.7% (IGBANG-D-2). Six accession namely IGBANG-D-2, IGKMFD-1, IG97-241, IG95-30 and IG95-114 exhibited maximum percent of increase in osmolality over their controls i.e., 247.7, 227.15, 221.3, 197.13 and 193.06 percent respectively (Table 16).

Osmotic potential (OP):

Osmotic potential was measured in control plants of 30 *Dichanthium* accessions where RWC ranged from 82.82 ± to 97.07% with an average of 91.87%. Osmotic potential at well irrigated (control) condition in all accessions ranged form -1.01± 0.006 Mpa (IGTGD-4) to -1.88± 0.070 (IG97-24) Mpa. with an average of 1.426±0.027 MPa. Water stress imposed by withholding the water to plants affected all water relation parameters significantly. A significant difference was observed in all the accessions under stressed plants (average RWC 49.38%) from their corresponding controls. Under water stress osmotic potential in 30 accessions ranged from -2.10± 0.010 to -4.15± 0.032MPa. Under moisture stress condition all accessions showed decrease in osmotic potential (more negative value). Minimum osmotic potential -4.15± 0.032 MPa was recorded in IGKMFD-1, where as maximum -2.10± 0.010 MPa was recorded in IG97-244. An average change of osmotic potential i.e., 1.63 MPa (ΔOP) was measured in all accessions from their respective control. Change in OP from control to stress (ΔOP) was observed in the range of 0.44 (IG97-244) to 2.92 (IGKMFD-1). Accession IGKMFD-1 showed remarkably higher decrease in osmotic potential i.e., ΔOP 2.92 MPa [from -1.27 (Control) to -4.15MPa in (stress)] under moisture stress (at RWC 52%). Whereas few others accessions like IG97-234, IG97-24, IG95-30,

Table 16: Change in osmolyte concentration in control and water stressed plants of 30 *Dichanthium* accessions.

Accession	mmole Kg^{-1} (control)	mmole Kg^{-1} (stress)	RWC (%) control	RWC (%) stress	(%) change in osmolality C to S
IG97-234	548.3 ±15.2	1410.0 ±9.09	90.60 ± 3.05	47.0 ± 1.25	157.1
IG97-24	752.0 ±23.1	1594.0 ±3.26	89.28± 0.91	52.58± 0.30	111.9
IG95-30	455.0 ±2.94	1276.0 ±9.41	92.21± 0.10	45.04± 0.04	197.13
IG97-192	453.6 ±3.09	1348.0 ±0.81	91.32± 1.09	48.02± 2.60	132
IG97-247	678.0 ±7.25	1378.3 ±1.24	89.72± 2.45	53.58± 2.53	103.29
IG97-241	432.0 ±8.60	1388.3 ±7.13	95.45± 0.07	47.57± 0.15	221.37
IG95-25	579.3 ±6.59	1226.3 ±20.6	92.25± 0.40	50.91± 1.02	111.68
IG95-114	504.3 ±2.49	1478.0 ±2.94	92.80± 1.59	48.31± 1.36	193.06
IG97-147	532.6 ±9.46	1127.0 ±6.68	92.01± 0.05	41.55± 2.29	111.57
IG97-170	705.0 ±10.0	1318.0 ±25.35	92.83± 0.89	58.11± 0.68	86.95
IG97-151	638.6 ±2.4	1425.3 ±46.94	93.58± 0.18	44.97± 0.41	123.17
IG97-152	573.6 ±2.8	1222.0 ±11.7	89.27± 0.72	43.21± 1.21	113.02
IG97-184	576.6 ±9.84	1254.3 ±7.40	90.0 ± 1.12	43.5.0± 1.70	117.51
IG97-158	554.6 ±2.49	1244.0 ±17.9	82.82± 0.14	44.45± 0.99	124.27
IG97-118	570.3 ±3.29	1266.6 ±10.6	92.39± 1.38	53.28± 0.14	122.09
IG97-233	597.6 ±8.05	1225.0 ±31.49	92.10± 2.12	55.81± 1.41	104.96
IG97-189	608.3 ±2.62	1250.3 ±26.5	90.00± 0.05	48.06± 0.94	105.53
IG97-132	655.3 ±13.5	1280.3 ±50.0	97.07± 1.61	47.68± 0.83	95.37
IG97-218	626.0 ±9.20	1323.0 ±4.96	95.25± 2.03	46.19± 1.39	111.34
IG97-130	661.6 ±9.97	1146.3 ±11.4	91.25± 1.66	43.29± 0.18	73.25
IG97-121	646.0 ±5.88	1038.3 ±39.3	92.46± 0.90	48.64± 0.82	60.73
IG97-244	66.03 ±11.8	840 .0 ±3. 26	96.69± 1.84	51.66± 1.15	26.69
IG97-245	524.6 ±2.86	849 .0 ±4.49	93.98± 0.47	50.66± 0.08	61.88
IG97-144	564.6 ±13.7	1039.6 ±28.6	93.09± 1.85	53.47± 1.13	84.12
IGKMFD-1	508.3 ±1.69	1663.0 ±10.6	91.83± 0.16	47.25± 1.58	227.15
IGTGD-4	404.3 ±2.05	1011.0 ±3.74	89.05± 0.28	55.36± 0.88	150.04
IGBANG-D-2	421.3 ±4.18	1465.0 ±21.7	92.48± 2.86	46.04± 0.91	247.70
IGKMD-10	479.0 ±29.4	1330.0 ±24.0	92.11± 0.03	46.78± 0.75	177.66
IG3108	525.0 ±38.8	900.0 ±8.16	90.12± 1.35	49.10± 2.28	71.43
IGBANG-D-1	579.6 ±3.39	996.3 ±6.64	92.15± 0.31	49.44± 2.87	71.89
Mean	**570.47±9.22**	**1243.8± 15.22**	**91.87± 0.34**	**49.38± 2.87**	**123.2**

* LSD at (P<0.05) 53.69

**LSD at (P<0.01) 61.73

IG97-241, IG97-114,IGBANG-D-2 and IGKMD-10 exhibited decrease in ΔOP and that ranged from 2.04 to 2.41MPa (Table 17) .

Water potential (WP):

Water potential was uniformly decreased (more negative) with increasing in magnitude of water stress. Water potential at well irrigated (control) condition in all accessions ranged form -1.06± 0.066 Mpa to -2.93± 0.133 MPa with an average of -1.80± 0.10 . Maximum WP -2.93± 0.133 MPa observed in IG97-218 whereas minimum (-1.06± 0.066MPa) observed in IG97-118. Under control condition out of 30 accessions, 5 accessions namely IG97-218, IG97-170, IG97-147, IG97-234, and IG97-233 showed water low water potential i.e., -2.93, -2.73, - 2.57, -2.29 and -2.20 MPa respectively. A group of five accessions namely IG97-192, IG97-247, IG97-241, IG95-25 and IG97-132 showed water potential -2.06 MPa in each under control. Another group of five accessions namely IG97-118, IGKMFD-1, IGTGD-4, IGBANG-D-2, and IGKMD-10 exhibited high water potential i.e., -1.06,-1.42, -1.24, -1.37 and -1.37MPa respectively under control among the accessions. Whereas rest of accessions showed moderate water potential (Table 18).

Water potential in 30 *Dichanthium* accessions decreased (more negative) from their corresponding control under water stress condition which ranged from –2.56 ± 0.200 to -6.02 ± 0.038 MPa. Mean water potential i.e., -4.06± 0.205 MPa was observed at water stress (at 49% RWC). Maximum WP -2.56± 0.200 MPa measured in IG97-158 whereas minimum (-6.02± 0.038 MPa) was observed in IG97-24. More than -5.0 MPa water potential (WP) was measured in 5 accessions namely. IG97-24, IG97-184, IG97-218, IG97-234 and IGBANG-D-2 where WP ranged from -5.02 to 6.02MPa with a mean value of -5.35 MPa. Five accessions viz. IG97-158, IG97-118, IGTGD-4, IGKMD-10 and IG3108 exhibited high water potential under stress i.e., -2.56,-2.71,-2.91,-2.59 and -2.93 MPa respectively with mean value -2.74 MPa. Rest of the accessions showed moderate water potential.

Table 17: Change in osmotic potential under control and water stressed plants of 30 *Dichanthium* accessions.

Accession	OP control (MPa)	OP stress (MPa)	RWC (%) control	RWC (%) stress	Change in OP from C to S ΔOP
IG97-234	-1.37±0.038	-3.52±0.027	90.60 ± 3.05	47.0 ± 1.25	2.15
IG97-24	-1.88±0.070	-3.99±0.010	89.28± 0.91	52.58± 0.30	2.10
IG95-30	-1.13±0.009	-3.37±0.002	92.21± 0.10	45.04± 0.04	2.23
IG97-192	-1.37±0.037	-3.19±0.028	91.32± 1.09	48.02± 2.60	1.81
IG97-247	-1.69±0.022	-3.44±0.003	89.72± 2.45	53.58± 2.53	1.75
IG97-241	-1.08±0.026	-3.47±0.021	95.45± 0.07	47.57± 0.15	2.41
IG95-25	-1.44±0.020	-3.06±0.063	92.25± 0.40	50.91± 1.02	1.56
IG95-114	-1.26±0.007	-3.69±0.009	92.80± 1.59	48.31± 1.36	2.42
IG97-147	-1.33±0.028	-2.81±0.020	92.01± 0.05	41.55± 2.29	1.46
IG97-170	-1.76±0.030	-3.29±0.077	92.83± 0.89	58.11± 0.68	1.45
IG97-151	-1.59±0.007	-3.56±0.143	93.58± 0.18	44.97± 0.41	1.96
IG97-152	-1.43±0.008	-3.05±0.036	89.27± 0.72	43.21± 1.21	1.59
IG97-184	-1.44±0.030	-3.13±0.022	90.0 ± 1.12	43.5.0± 1.70	1.71
IG97-158	-1.38±0.007	-3.11±0.055	82.82± 0.14	44.45± 0.99	1.72
IG97-118	-1.42±0.010	-3.16±0.032	92.39± 1.38	53.28± 0.14	1.77
IG97-233	-1.49±0.024	-3.06±0.096	92.10± 2.12	55.81± 1.41	1.49
IG97-189	-1.52±0.008	-3.12±0.081	90.00± 0.05	48.06± 0.94	1.60
IG97-132	-1.63±0.041	-3.20±0.153	97.07± 1.61	47.68± 0.83	1.69
IG97-218	-1.56±0.028	-3.30±0.015	95.25± 2.03	46.19± 1.39	1.72
IG97-130	-1.65±0.030	-2.86±0.035	91.25± 1.66	43.29± 0.18	1.25
IG97-121	-1.62±0.018	-2.59±0.120	92.46± 0.90	48.64± 0.82	1.12
IG97-244	-1.66±0.036	-2.10±0.010	96.69± 1.84	51.66± 1.15	0.44
IG97-245	-1.31±0.008	-2.12±0.013	93.98± 0.47	50.66± 0.08	0.81
IG97-144	-1.41±0.042	-2.59±0.087	93.09± 1.85	53.47± 1.13	1.28
IGKMFD-1	-1.27±0.005	-4.15±0.032	91.83± 0.16	47.25± 1.58	2.92
IGTGD-4	-1.01±0.006	-2.52±0.011	89.05± 0.28	55.36± 0.88	1.51
IGBANG-D-2	-1.05±0.012	-3.06±0.066	92.48± 2.86	46.04± 0.91	2.54
IGKMD-10	-1.19±0.090	-3.32±0.073	92.11± 0.03	46.78± 0.75	2.04
IG3108	-1.31±0.119	-2.25±0.025	90.12± 1.35	49.10± 2.28	0.93
IGBANG-D-1	-1.45±0.010	-2.49±0.020	92.15± 0.31	49.44± 2.87	1.05
Mean	**-1.43±0.027**	**-3.11±0.040**	**91.87±0.34**	**49.3± 2.87**	**1.68**

* LSD at (P<0.05) 0.134

**LSD at (P<0.01) 0.175

Difference between water potential of control and stressed plants of the accessions (ΔWP) was also calculated. Among all the accessions the mean ΔWP was recorded as -2.26 MPa. The ΔWP observed among the accessions ranged from -0.71 to -4.75 MPa. Maximum decrease in water potential form control to stress (ΔWP) was exhibited by accession IG97-24, i.e., -4.57 MPa on the other hand minimum ΔWP was in IG97-118 i.e., -0.71 MPa. Out of 30, 7 accessions namely IG97-24, IGBANG-D-2, IG97-151, IG97-121, IG95-30, IG97-114 and IG97-130 exhibited high decrease in water potential (more negative) from their respective controls. The ΔWP of these accessions were -4.57, -3.64, -3.32, -3.26, -3.22, -3.00, and -3.00 Mpa respectively with mean ΔWP of -3.43 MPa. Apart form accession IG97-158, three other accessions viz., IG3108, IG97-147 and IGKMD-10 showed less ΔWP i.e, -1.42, -1.08 and -1.22 MPa respectively. Among the 30 accessions, 18 accessions showed ΔWP in the range of -1.55 to -2.92MPa, with an average of -1.99 MPa (Table 18).

Osmotic adjustment (OA):

Osmotic potential at full turgor (OP_{100}) was calculated from osmotic potential recorded under control and stressed plants of 30 *Dichanthium* accessions. OP_{100} under control level was ranged from -0.98 ±0.006 to -1.84± 0.070 MPa with an average value of -1.43± 0.027 MPa. The maximum OP_{100} i.e., -0.98 ±0.006 MPa was observed in IGTGD-4 where as minimum -1.84± 0.070 MPa in IG97-24. The osmotic potential at full turgor (OP_{100}) under stress condition was ranged from -1.38± 0.063 to -2.74± 0.020 MPa. The mean OP_{100} under stress condition was -1.72± 0.052MPa. The minimum OP_{100} i.e., -2.74±0.020 MPa was observed in IG97-24 and maximum -1.38± 0.063 MPa in IG97-121. Osmotic adjustment capacity of accession was determined as a difference between osmotic potential at full turgor (OP_{100}) in control and stressed plants. Osmotic adjustment among the accession ranged from 0.05± 0.088 to 0.896± 0.070 MPa under water stress condition. The mean OA recorded in *Dichanthium* accession at stress condition among the accessions was 0.295 ±0.069 (49% RWC). Maximum osmotic

Table 18: Change in water potential (WP) under control and water stressed plants of 30 *Dichanthium* accessions.

Accession	WP control (C) (MPa)	WP stress (S) (MPa)	RWC (%) control	RWC (%) stress	Change in WP from C to S (ΔWP)
IG97-234	-2.29±0.23	-5.22±0.04	90.60 ± 3.05	47.0 ± 1.25	2.92
IG97-24	-1.44±0.04	-6.02±0.04	89.28± 0.91	52.58± 0.30	4.57
IG95-30	-1.71±0.10	-4.93±0.13	92.21± 0.10	45.04± 0.04	3.22
IG97-192	-2.10±0.10	-4.60±0.20	91.32± 1.09	48.02± 2.60	2.50
IG97-247	-2.06±0.07	-4.31±0.18	89.72± 2.45	53.58± 2.53	2.25
IG97-241	-2.06±0.07	-3.93±0.20	95.45± 0.07	47.57± 0.15	1.86
IG95-25	-2.06±0.07	-3.92±0.13	92.25± 0.40	50.91± 1.02	1.85
IG95-114	-1.46±0.07	4.46±0.07	92.80± 1.59	48.31± 1.36	3.00
IG97-147	-2.57±0.21	-3.66±0.13	92.01± 0.05	41.55± 2.29	1.08
IG97-170	-2.73±0.13	-4.53±0.20	92.83± 0.89	58.11± 0.68	1.80
IG97-151	-1.50±0.10	-4.82±0.78	93.58± 0.18	44.97± 0.41	3.32
IG97-152	-1.93±0.07	-4.00±0.40	89.27± 0.72	43.21± 1.21	2.06
IG97-184	-1.90±0.30	-5.26±0.07	90.0 ± 1.12	43.5.0± 1.70	3.36
IG97-158	-1.55±0.10	-2.26±0.20	82.82± 0.14	44.45± 0.99	0.71
IG97-118	-1.06±0.07	-2.71±0.17	92.39± 1.38	53.28± 0.14	1.64
IG97-233	-2.20±0.20	-4.84±0.34	92.10± 2.12	55.81± 1.41	2.64
IG97-189	-1.77±0.10	-3.33±0.27	90.00± 0.05	48.06± 0.94	1.55
IG97-132	-2.06±0.20	-4.11±0.30	97.07± 1.61	47.68± 0.83	2.04
IG97-218	-2.93±0.13	-5.26±0.20	95.25± 2.03	46.19± 1.39	2.33
IG97-130	-1.79±0.33	-4.80±0.00	91.25± 1.66	43.29± 0.18	3.00
IG97-121	-1.43±0.14	-4.70±0.10	92.46± 0.90	48.64± 0.82	3.26
IG97-244	-1.62±0.34	-3.19±0.18	96.69± 1.84	51.66± 1.15	1.57
IG97-245	-1.33±0.12	-3.04±0.51	93.98± 0.47	50.66± 0.08	1.71
IG97-144	-1.66±0.33	-3.26±0.20	93.09± 1.85	53.47± 1.13	1.59
IGKMFD-1	-1.42±0.10	-3.24±0.203	91.83± 0.16	47.25± 1.58	1.82
IGTGD-4	-1.24±0.13	-2.91±0.407	89.05± 0.28	55.36± 0.88	1.66
IGBANG-D-2	-1.37±0.10	-5.02±0.222	92.48± 2.86	46.04± 0.91	3.64
IGKMD-10	-1.37±0.19	-2.59±0.066	92.11± 0.03	46.78± 0.75	1.22
IG3108	-1.51±0.31	-2.93±0.176	90.12± 1.35	49.10± 2.28	1.42
IGBANG-D-1	-1.93±0.13	-4.13±0.067	92.15± 0.31	49.44± 2.87	2.20
Mean	**1.805±0.15**	**4.06±0.205**	**91.87± 0.34**	**49.38 ± 2.78**	**2.26**

* LSD at (P<0.05) 0.223

** LSD at (P<0.01) 0.292

adjustment 0.896 ± 0.070MPa was observed in IG97-24 whereas minimum OA 0.05 ± 0.088 MPa in IG97-218. Out of 30, 9 accessions significantly showed higher OA [0.515 +0.015 (IG95-30) to 0.896 ±0.070MPa (IG97-24)]. These accessions Viz., IG97-24, IGKMFD-1, IGBANG-D-2, IG97-233, IG97-241, IG95-114, IG97-234, IGTGD-4 and IG95-30 exhibited OA i.e., 0.896, 0.886, 0.723, 0.706, 0.693, 0.676, 0.590, 0.546 and 0.515 MPa respectively. Moderate OA value obtained in 8 accessions and that ranged from 1.98 ± 0.025 (IG97-184) to 0.400± 0.055 MPa (IG97-118). The least OA value was obtained from 10 accessions and that ranged from 0.050+ 0.088 IG97-218) to 0.163+0.120 (IG3108).Three accessions namely IG97-130, IG97-121 and IG97-244 exhibited negative OA at this stress level (Table 19).

Injury index (I):

Membrane injury as a consequence of water stress was measured in form of electrolyte leakage and denoted as injury index. The injury index was measured at maximum stress condition in 30 accessions, where average RWC was 49%.The injury index obtained among the 30 *Dichanthium* accessions ranged from 3.35% to 23.63%. The average injury index obtained among the accessions was 11.24% when severe water stress was imposed. The least injury index (3.35%) was observed in IGBANG-D-2 whereas maximum (23.63%) was observed in IGTGD-4. Out of 30, 2 accessions namely IGTGD-4 and IG3108 showed highest injury index (i.e., 23.63 and 20.0% respectively). Other accessions namely IG97-147, IG97-118, IG97-132, IG97-144, IG97-151, IG97-152, IG97-158 and IGKMFD-1 showed higher injury index viz., 16.43, 15.25, 14.28, 14.21, 14.12, 14.12, 15.04 and 13.19% respectively with an average value of 14.51% injury index. A set of 8 accessions exhibited significantly low injury index and that ranged from 3.5 ± 0.25 to 8.54 ± 0.60, with an average value of 6.16. These accessions were IGBANG-D-2, IG97-24, IGKMD-10, IG95-30, IG97-192, IG97-247, IG97-233, IGBANG-D-1 and IG97-234 and showed injury index of 3.5, 3.6, 3.8, 5.7, 6.8, 7.0, 8.24, 8.31 and 8.54 respectively (Table 19).

Table 19: OP_{100} in control and stressed plants osmotic adjustment and Injury index in 30 *Dichanthium* accessions.

Accession No	OP100 Control	OP100 Stress	OA	Injury index
IG97-234	-1.23 ±0.038	-1.82 ±0.011	0.590 ±0.023	8.54 ±0.60
IG97-24	-1.84 ±0.070	-2.74 ±0.020	0.896 ±0.070	3.69 ±0.19
IG95-30	-1.14 ±0.009	-1.66 ±0.005	0.515 ±0.015	5.70 ±0.20
IG97-192	-1.38 ±0.037	-1.69 ±0.015	0.310 ±0.048	6.78 ±0.30
IG97-247	-1.67 ±0.022	-2.02 ±0.001	0.359 ±0.033	7.16 ±0.16
IG97-241	-1.13 ±0.026	-1.82 ±0.032	0.693 ±0.020	10.62±0.27
IG95-25	-1.46 ±0.020	-1.71 ±0.070	0.243 ±0.075	12.34±0.84
IG95-114	-1.28 ±0.007	-1.96 ±0.060	0.676 ±0.085	10.94±0.34
IG97-147	-1.31 ±0.028	-1.43 ±0.009	0.107 ±0.019	16.43±0.90
IG97-170	-1.81 ±0.030	-2.10 ±0.043	0.283 ±0.050	12.92±0.34
IG97-151	-1.64 ±0.007	-1.75 ±0.066	0.113 ±0.070	14.12±1.12
IG97-152	-1.40 ±0.008	-1.51 ±0.017	0.115 ±0.037	14.12±0.43
IG97-184	-1.42 ±0.030	-1.62 ±0.004	0.198 ±0.025	12.34±0.30
IG97-158	-1.26 ±0.007	-1.51 ±0.005	0.256 ±0.020	15.04±0.31
IG97-118	-1.43 ±0.010	-1.85 ±0.015	0.400 ±0.055	15.25±0.65
IG97-233	-1.50 ±0.024	-2.21 ±0.110	0.706 ±0.055	8.24 ±0.25
IG97-189	-1.50 ±0.008	-1.65 ±0.010	0.150 ±0.017	9.87 ±0.36
IG97-132	-1.62 ±0.041	-1.68 ±0.080	0.057 ±0.115	14.28±0.64
IG97-218	-1.62 ±0.028	-1.67 ±0.050	0.050 ±0.088	10.73±1.26
IG97-130	-1.65 ±0.030	-1.43 ±0.150	-0.220 ±0.127	11.51±0.45
IG97-121	-1.64 ±0.018	-1.38 ±0.063	-0.253 ±0.047	9.59 ±0.60
IG97-244	-1.76 ±0.036	-1.42 ±0.030	-0.340 ±0.087	9.56 ±0.66
IG97-245	-1.35 ±0.008	-1.41 ±0.005	0.063 ±0.015	10.90±0.90
IG97-144	-1.41 ±0.042	-1.52 ±0.060	0.113 ±0.049	14.21±0.66
IGKMFD-1	-1.27 ±0.005	-2.15 ±0.077	0.886 ±0.087	13.19±0.23
IGTGD-4	-0.98 ±0.006	-1.53 ±0.035	0.546 ±0.035	23.63±1.45
IGBANG-D-2	-1.06 ±0.012	-1.78 ±0.120	0.723 ±0.165	3.53 ±0.25
IGKMD-10	-1.20 ±0.090	-1.49 ±0.361	0.286 ±0.423	3.80 ±0.20
IG3108	-1.29 ±0.119	-1.46 ±0.016	0.163 ±0.120	20.00±1.32
IGBANG-D-1	-1.46 ±0.010	-1.61 ±0.013	0.153 ±0.010	8.31 ±1.02
Mean	**-1.43 ±0.027**	**-1.72 ±0.052**	**0.295 ±0.069**	**11.25± 0.57**

* LSD at (P<0.05) 0.078

** LSD at (P<0.01) 0.102

Enzyme activities:

Peroxidase enzyme (POD) activity:

Peroxidase activity was measured in 30 *Dichanthium* accessions at control (well irrigated) and water stress (withholding water) condition and expressed in units mg protein^{-1} min^{-1}. The status and magnitude of water stress was determined by measuring the relative water content (RWC). POD activity obtained in 30 *Dichanthium* the accessions which did not give any regular and uniform pattern. Enzyme activity measured under control in 30 *Dichanthium* accessions at RWC ranged from 82.82 to 97.07% with an average of 91.87% (Table 20). Peroxidase activity in control plants ranged from 2.09± 0.213 (IG97-118) to 52.70± 0.566 (IGTGD-4) units mg protein^{-1} min^{-1}. The mean enzyme activity in control plants was15.69 units mg protein^{-1} min^{-1}. The maximum enzyme activity (52.70±0.566 units mg protein^{-1} min^{-1}) was observed in IGTGD-4 and least activity (2.09± 0.213) in IG97-118. Out of 30, 9 accessions gave higher enzyme activity in under control condition that ranged from 20.07± 0.365 (IG97-121) to 52.70± 0.566 (IGTGD-4) units mg protein^{-1} min^{-1}. A group of six accessions namely IG97-118, IG97-132, IGBANG-D-2, IG97-170, IG97-218 and IG97-147 exhibited less enzyme activity i.e., 2.09, 2.38, 4.69, 4.71, 5.02 and 6.18 units mg protein^{-1} min^{-1} respectively with an average of 4.17 units mg protein^{-1} min^{-1}. Rest of accessions showed moderate enzyme activity that ranged from 7.08± 0.213 (IG97-158) to 17.54± 0.285 (IG97-151) units mg protein^{-1} min^{-1}, with an average of 12.59 units mg protein^{-1} min^{-1}.

Water stress condition as determined by RWC ranged from 41.0±2.29 to 61.0±1.15 % with an average of 49.38 %. Accessions IG97-234, IG97-24 and IG95-30 took maximum days (16) to reach the average level of RWC i.e., 48.0% where as accessions IGTGD-4, IGBANG-D-2, IGKMD-10, IG3108 and IGBANG-D-1 took minimum 6-7 days to reach (49%) RWC level. Rest of the accessions reached to this level in 8-13 days. At this level of water stress the

Table 20: Peroxidase enzyme specific activity (units mg protein^{-1} min^{-1}) of *Dichanthium* accessions under control and stress condition.

Accession	unit/mg protein control	unit/mg protein stress	RWC (%) control	RWC (%) stress	% change in POD C to S
IG97-234	26.01± 1.40	23.92± 3.63	90.60 ± 3.05	47.0 ±1.25	-8.0
IG97-24	25.72± 3.65	30.92± 0.68	89.28± 0.91	62.58± 0.30	20.1
IG95-30	24.32± 0.90	21.09± 2.15	92.21± 0.10	45.04± 0.04	-13.2
IG97-192	29.06± 0.88	10.63± 0.99	91.32± 1.09	48.02± 2.60	-63.4
IG97-247	36.54± 0.78	42.19± 6.41	89.72± 2.45	53.58± 2.53	15.4
IG97-241	20.75± 0.39	17.26± 1.76	95.45± 0.07	47.57± 0.15	-16.8
IG95-25	12.95± 0.39	12.30± 0.19	92.25± 0.40	50.91± 1.02	-5.0
IG95-114	9.68± 0.45	8.15± 0.20	92.80± 1.59	48.31± 1.36	-15.7
IG97-147	6.18± 0.85	4.87± 0.39	92.01± 0.05	41.55± 2.29	-21.2
IG97-170	4.71 ± 0.18	14.04± 0.08	92.83± 0.89	58.11± 0.68	197.9
IG97-151	17.54± 2.85	11.20± 0.51	93.58± 0.18	44.97± 0.41	-36.1
IG97-152	14.58± 0.65	15.28± 0.78	89.27± 0.72	43.21± 1.21	4.7
IG97-184	10.50± 0.88	14.40± 0.17	90.0 ± 1.12	43.5.0± 1.70	37.1
IG97-158	7.08± 0.57	10.07± 0.90	82.82± 0.14	44.45± 0.99	42.0
IG97-118	2.09± 0.21	5.62± 0.36	92.39± 1.38	53.28± 0.14	168.8
IG97-233	7.36± 0.95	9.87± 0.19	92.10± 2.12	65.81± 1.41	34.2
IG97-189	9.93± 0.82	8.61± 0.57	90.00± 0.05	48.06± 0.94	-13.2
IG97-132	2.38± 0.21	2.53± 0.17	97.07± 1.61	47.68± 0.83	6.4
IG97-218	5.02 ± 0.61	6.28± 0.48	95.25± 2.03	46.19± 1.39	25.0
IG97-130	13.06± 1.15	13.45± 0.49	91.25± 1.66	43.29± 0.18	-16.2
IG97-121	20.0±7 0.36	9.60± 0.14	92.46± 0.90	48.64± 0.82	-52.1
IG97-244	15.78± 0.69	15.59± 0.13	96.69± 1.84	61.66± 1.15	-1.2
IG97-245	15.61± 0.63	17.66± 0.13	93.98± 0.47	60.66± 0.08	13.1
IG97-144	10.62± 0.99	11.65± 0.22	93.09± 1.85	53.47± 1.13	9.6
IGKMFD-1	13.0± 0.37	11.66± 0.36	91.83± 0.16	47.25± 1.58	-10.2
IGTGD-4	52.70± 0.56	79.30± 3.79	89.05± 0.28	55.36± 0.88	50.4
IGBANG-D-2	4.69± 0.74	8.32± 1.66	92.48± 2.86	46.04± 0.91	77.4
IGKMD-10	17.23± 2.07	34.07± 0.80	92.11± 0.03	46.78± 0.75	97.7
IG3108	21.60± 0.73	12.30± 0.55	90.12± 1.35	49.10± 2.28	-43.0
IGBANG-D-1	11.09± 0.38	7.44± 0.22	92.15± 0.31	49.44± 2.87	-32.8
Mean	**15.69±0.63**	**16.34±0.83**	**91.87±0.310**	**49.38±2.87**	

* LSD at (P<0.05) 3.81

**LSD at (P<0.01) 5.01

pattern of peroxidase activity was not seen as regular increase or decrease in 30 *Dichanthium* accessions. A pair wise T tests for significance difference between control and stress indicated the significance difference in all accession except IG97-234 and IG97-244. PRX activity under stress among the *Dichanthium* accessions ranged from 2.53±0178 (IG97-132) to 79.30±3.70 (IGTGD-4) units mg protein^{-1} min^{-1}, with a mean value of 16.34 units mg protein^{-1} min^{-1}. The maximum peroxidase activity (79.30 ± 3.79 units mg protein^{-1} min^{-1}) under water stressed condition was observed in IGTGD-4 whereas minimum (2.53± 0.178 units mg protein^{-1} min^{-1}) in IG97-132. Out of 30 accessions, 15 showed increases in enzyme activity (specific activity) and rest of the accession showed decrease in activity. Fifteen accessions which showed increase in specific activity ranged from 4.7 to 168 % over their corresponding control. Maximum (197 %) increase in peroxidase activity was observed in accession IG97-170 and minimum 4.7% in IG97-152 with their respective control.

Superoxide dismutase (SOD) enzyme activity:

Superoxide dismutase enzyme activity was measured in 30 *Dichanthium* accessions under control (well irrigated) and water stress (withholding water) condition and represented as units mg protein^{-1} min^{-1}. Enzyme activity in stressed plants was measured at higher magnitude of water stress. The status and magnitude of water stress was determined by measuring the relative water content (RWC). Superoxide dismutase (SOD) activity measured at control condition (mean RWC 91.87%) indicated accession wise variation in activity. It ranged from 0.116± 0.11 (IG97-118) to 0.626± 0.004 (IG95-30) units mg protein^{-1} min^{-1} with average value of 0.391 units mg protein^{-1} min^{-1} among the accessions. Out of 30, 10 accessions namely IG97-234, IG97-24, IG97-151, IG95-30, IG97-192, IG97-247, IG97-241, IG95-114, IGTGD-4 and IGKMD-10 showed higher enzyme activity, i.e., 0.518, 0.477, 0.481, 0.626, 0.548, 0.519, 0.480, 0.531, 0.562, and 0.564 units mg protein^{-1} min^{-1} respectively, with an average of 0.530 units mg protein^{-1} min^{-1}. Activity among these accessions ranged from 0.477±

0.016 (IG97-247) to 0.626 ± 0.004 (IG95-30) units mg protein^{-1} min^{-1}, whereas in rest of the accessions it ranged from 0.116± 0.11 (IG97-118) to 0.458± 0.031 (IG97-184) units mg protein^{-1} min^{-1} (Table 21)..

When water stress was imposed by withholding the water relative water content (RWC) dropped to an average of 91.87 to 49.38%. SOD activity obtained at this stage ranged from 0.141± 0.003 (IG3108) to 0.700 ±0.001(IGTGD-4) units mg protein^{-1} min^{-1} among the accessions with average of 0.290 units mg protein^{-1} min^{-1}. Among the 30, 27 accessions exhibited decrease in superoxide dismutase activity where as three accessions namely IG97-118, IGKMFD-1 and IGTGD-4 exhibited increase in activity at this level of water stress. The increase in enzyme activity observed in IG97-118 i.e., from 0.116± 0.11 (control) to 0.161± 0.001 (stress) which accounts for 37.7% increase over its corresponding control and in IGTGD and IGKMFD-1 accessions where activity increased by 4.7 and 23.8 % respectively over their control respectively. The activity obtained from rest of 27 accessions ranged from 0.141± 0.003 (IG3108) to 0.553 ± 0.001(IGKMD-10) units mg protein^{-1} min^{-1} with average of 0.277 units mg protein^{-1} min^{-1}. Among the 27 accessions where decrease in activity was observed it ranged from 1.4 (IGBANG-D-2) to 58.3% (IG95-30) over their control. Four accessions namely, IG95-30, IG97-121, IG3108 and IGBANG-D-1 exhibited more than 50% decrease in enzyme activity from their respective controls and that ranged from 52 (IG3108) to 58.3% (IG95-30) with an average decrease of 54.54%. .Nine accessions namely IG97-234, IG97-241, IG97-151, IG97-152, IG97-184, IG97-233, IG97-189, IG97-245 and IG97-144, exhibited comparatively less decrease in enzyme activity i.e., 35.2, 40.5, 47.1, 33.5, 40.7, 36.45, 34.1, 46.8, and 48.05 % respectively. It ranged from 33.5 to 48.05% with an average of 40.26%. Five accessions namely IG97-24, IG95-25,IG97170, IGBANG-D-2 and IGKMD-10 showed insignificant decrease in activity at LSD P<0.05 and 0.01 whereas other 9 accessions which showed least decreased in SOD activity under stress ranged from 12.9 (IG97-247) to 29.9%(IG97-147) with an average of 20.4% (Table 21).

Table 21: Superoxide dismutase enzyme specific activity in units mg protein^{-1} min^{-1} in 30 *Dichanthium* accessions under control and stress condition.

Accession	unit/mg protein/min control	RWC (%) control	unit/mg protein/min stress	RWC (%) stress	% change
IG97-234	0.518±0.02	90.60 ± 3.05	0.335±0.085	47.0 ±1.25	-35.26
IG97-24	0.477±0.02	89.28± 0.91	0.446±0.002	52.58± 0.30	-6.34
IG95-30	0.626 ±0.04	92.21± 0.10	0.261±0.002	45.04± 0.04	-58.36
IG97-192	0.548±0.03	91.32± 1.09	0.406±0.068	48.02± 2.60	-25.87
IG97-247	0.519±0.04	89.72± 2.45	0.452±0.040	53.58± 2.53	-12.90
IG97-241	0.480±0.10	95.45± 0.07	0.285±0.058	47.57± 0.15	-40.53
IG95-25	0.277±0.04	92.25± 0.40	0.257±0.002	50.91± 1.02	-7.347
IG95-114	0.531±0.02	92.80± 1.59	0.385±0.005	48.31± 1.36	-27.52
IG97-147	0.303±0.02	92.01± 0.05	0.214±0.003	41.55± 2.29	-29.23
IG97-170	0.284±0.05	92.83± 0.89	0.273±0.014	58.11± 0.68	-3.826
IG97-151	0.481±0.04	93.58± 0.18	0.254±0.031	44.97± 0.41	-47.19
IG97-152	0.431±0.02	89.27± 0.72	0.287±0.001	43.21± 1.21	-33.50
IG97-184	0.458±0.03	90.0 ± 1.12	0.271±0.101	43.5.0± 1.70	-40.70
IG97-158	0.271±0.01	82.82± 0.14	0.227±0.003	44.45± 0.99	-16.38
IG97-118	0.116±0.01	92.39± 1.38	0.161±0.001	53.28± 0.14	37.71
IG97-233	0.362±0.02	92.10± 2.12	0.230±0.002	55.81± 1.41	-36.45
IG97-189	0.383±0.03	90.00± 0.05	0.252±0.002	48.06± 0.94	-34.17
IG97-132	0.233±0.04	97.07± 1.61	0.187±0.002	47.68± 0.83	-19.85
IG97-218	0.296±0.02	95.25± 2.03	0.251±0.003	46.19± 1.39	-15.33
IG97-130	0.300±0.02	91.25± 1.66	0.256±0.004	43.29± 0.18	-14.75
IG97-121	0.442±0.05	92.46± 0.90	0.206±0.004	48.64± 0.82	-53.26
IG97-244	0.368±0.06	96.69± 1.84	0.286±0.001	51.66± 1.15	-22.22
IG97-245	0.364±0.04	93.98± 0.47	0.193±0.004	50.66± 0.08	-46.82
IG97-144	0.366±0.08	93.09± 1.85	0.190±0.006	53.47± 1.13	-48.05
IGKMFD-1	0.286±0.01	91.83± 0.16	0.299±0.003	47.25± 1.58	4.76
IGTGD-4	0.565±0.05	89.05± 0.28	0.700±0.001	55.36± 0.88	23.88
IGBANG-D-2	0.254±0.01	92.48± 2.86	0.251±0.006	46.04± 0.91	-1.41
IGKMD-10	0.564±0.02	92.11± 0.03	0.553±0.001	46.78± 0.75	-1.97
IG3108	0.296±0.01	90.12± 1.35	0.141±0.003	49.10± 2.28	-52.03
IGBANG-D-1	0.324±0.05	92.15± 0.31	0.148±0.002	49.44± 2.87	-54.33
Mean	**0.391±0.003**	**91.87±0.34**	**0.290±0.34**	**49.38±02.87**	**-23.97**

* LSD (P<0.05) 0.0361
**LSD (P<0.01) 0.0477

Pyrroline 5-carboxylate synthetase (P5CS) enzyme activity:

Pyrroline 5-carboxylate synthetase (P5CS) enzyme activity was measured in 30 *Dichanthium* accessions at control (well irrigated) and water stress (withholding water) condition and represented in µmoles min.$^{-1}$. Enzyme activity in stressed plants was measured at maximum stress condition as indicated by relative water content (RWC) of the leaves. P5CS activity was determined in µmoles min^{-1}. P5CS activity measured at control condition (mean RWC 90.0%) in 30 *Dichanthium* accessions. The variation in enzyme activity at control condition was observed and that ranged from 0.170± 0.211 (IGTGD-4) to 5.42 ± 2.00 (IG97-152) µmoles min^{-1} with an average value of 2.51 µmoles min^{-1}. The maximum enzyme activity 5.42 ±2.00 µmoles min^{-1} was observed in IG97-152 and minimum 0.170± 0.211 µmoles min^{-1} in IGTGD-4. Out of 30, 5 accessions namely, IG97-152, IG97-241, IG97-245, IG97-147 and IG95-30 exhibited high enzyme activity i.e., 5.41 ± 0.50, 4.72± 0.374, 4.52 ± 0.930, 4.12 ± 0.105 and 4.03 ± 0.328 µmoles min^{-1} respectively with an average of 4.56 µmoles min^{-1} (Table 22).

Twenty one accessions exhibited enzyme activity in the range of 1.08± 0.034 (IG97-158) to 3.97 ± 0.037 (IG97-151) µmoles min^{-1}. Four accessions namely IGTGD-4, IGBANGD-1, IGKMFD-1 and IG3108 exhibited least enzyme activity i.e., 0.170, 0.173, 0.225 and 0.407 respectively. It ranged form 0.170± 0.211 (IGTGD-4) to 0.407 ±0.054 (IG3108) µmoles min^{-1}.

Under water stress condition (mean RWC 49%) enzyme activity of individual accession was measured. A pair wise T tests for significance difference in enzyme activity between control and stress plants of individual accession indicated significance difference in all accession except IG97-247. Out of 30, 28 accessions showed increase in enzyme activity whereas 2 accessions showed decrease in activity when compared with their respective control. P5CS activity under water stress was ranged from 0.19± 0.005 (IG3108) to 34.63±5.80 (IG97-

Table 22: P5CS enzyme activity in µmole min^{-1} of *Dichanthium* accessions under control and stress condition.

Accession	µmole min^{-1} control	µmole min^{-1} stress	RWC (%) control	RWC (%) stress	Fold change
IG97-234	2.94 ±0.79	9.08±0.45	90.60 ± 3.05	47.0 ±1.25	2.08
IG97-24	1.59±0.24	1.41±0.15	89.28± 0.91	52.58± 0.30	-0.11
IG95-30	4.01±0.33	4.41±0.38	92.21± 0.10	45.04± 0.04	0.09
IG97-192	1.63±0.26	5.03±0.15	91.32± 1.09	48.02± 2.60	2.09
IG97-247	1.88±0.39	8.13±0.35	89.72± 2.45	53.58± 2.53	3.31
IG97-241	4.72±0.37	11.87±2.00	95.45± 0.07	47.57± 0.15	-0.60
IG95-25	3.21±0.03	4.54±0.20	92.25± 0.40	50.91± 1.02	0.41
IG95-114	2.88±0.22	4.58±0.28	92.80± 1.59	48.31± 1.36	0.58
IG97-147	4.12±0.11	34.63±5.88	92.01± 0.05	41.55± 2.29	7.40
IG97-170	3.02±0.12	10.42±2.00	92.83± 0.89	58.11± 0.68	2.45
IG97-151	3.97±0.04	8.32±0.47	93.58± 0.18	44.97± 0.41	1.09
IG97-152	5.41±0.50	9.08±0.27	89.27± 0.72	43.21± 1.21	0.67
IG97-184	3.79±0.24	10.76±0.30	90.0 ± 1.12	43.5.0± 1.70	1.84
IG97-158	1.08±0.03	5.75±0.79	82.82± 0.14	44.45± 0.99	4.32
IG97-118	2.13±0.13	2.99±0.28	92.39± 1.38	53.28± 0.14	0.40
IG97-233	1.47±0.04	3.81±0.68	92.10± 2.12	55.81± 1.41	1.58
IG97-189	2.85±0.47	11.75±1.60	90.00± 0.05	48.06± 0.94	3.11
IG97-132	1.39±0.29	3.50±0.15	97.07± 1.61	47.68± 0.83	1.50
IG97-218	3.33±0.80	10.94±0.66	95.25± 2.03	46.19± 1.39	2.28
IG97-130	1.12±0.07	6.34±0.27	91.25± 1.66	43.29± 0.18	4.66
IG97-121	2.37±0.27	2.77±0.23	92.46± 0.90	48.64± 0.82	0.16
IG97-244	2.51±0.24	3.07±0.06	96.69± 1.84	51.66± 1.15	0.28
IG97-245	4.52±0.93	8.11±0.12	93.98± 0.47	50.66± 0.08	-0.44
IG97-144	3.34±0.29	5.50±0.17	93.09± 1.85	53.47± 1.13	0.64
IGKMFD-1	0.22±0.04	4.77±0.49	91.83± 0.16	47.25± 1.58	20.2
IGTGD-4	0.17±0.21	1.513±0.32	89.05± 0.28	55.36± 0.88	1.13
IGBANG-D-2	2.46±0.29	5.01±0.86	92.48± 2.86	46.04± 0.91	1.03
IGKMD-10	2.18±0.07	3.04±0.21	92.11± 0.03	46.78± 0.75	0.39
IG3108	0.40±0.05	0.19±0.01	90.12± 1.35	49.10± 2.28	-0.52
IGBANG-D-1	0.17±0.02	2.24±0.13	92.15± 0.31	49.44± 2.87	11.91
Mean	**2.51±0.26**	**6.78±0.66**	**91.87± 0.34**	**49.38±2.87**	**2.91**

* LSD at (P<0.05) 1.37 * LSD at (P<0.05) 1.45
** LSD at (P<0.01) 1.81 ** LSD at (P<0.01) 1.90

147) µmoles min^{-1} with mean value of 6.79± 0.667µmoles min^{-1}. Out of 30, 28 exhibited increase in SOD activity, where IGKMFD-1 showed maximum increase in enzyme activity i.e., 0.225 µmoles min^{-1} (control) to 4.77 µmoles min^{-1} activity (stress), which accounts for 21.2 fold increase over its control. Minimum increase was observed in IG95-30 i.e., from 4.013 (control) to 4.41 µmoles min^{-1} (stress), which accounts for 0.09 fold increase. Among the 28 accessions a group of 11 accessions namely, IGKMFD-1, IGBANG-D-1, IG97-147, IG97-130, IG97-158, IG97-247, IG97-189, IG97-170, IG97-218, IG97-192, and IG97-234 exhibited i.e., 22.2, 11.9, 7.4, 4.66, 4.32, 3.31, 3.11, 2.45, 2.28, 2.09 and 2.08 folds increase respectively, ~~with an average of 5.8fold increase~~. The decrease in enzyme activity was observed in two accessions namely IG97-24, IG3108, over their control.

Molecular markers association study:

Regression analysis was conducted using the data of stress responsive traits and data of three molecular marker systems viz; RAPD, STS, and ISSR. In total eight stress parameters were used for association in all 30 *Dichanthium* accessions. These parameters are ~~namely~~, Osmotic adjustment (OA), osmolality, MDA content, proline content, injury index, peroxidase activity, superoxide activity, and P5CS activity. Association of RAPD, STS and ISSR markers with these eight stress responsive traits were investigated using multiple regression analysis.

Association of RAPD marker:

In total 56 RAPD primers were tested for association with eight stress responsive traits. Out of 56 primers, 26 primers showed association with one or more stress responsive traits. Among these primers one or more bands were found to be associated with one or more traits. The association was studied at two confidence levels i.e., 99% (0.01) and at 99.9% (0.001) (Table 23).

Among 26 primers , 12 primers namely;OPQ-09 (570bp), OPR-04 (500bp), OPR-06 (850bp), OPF-01 (1000bp) OPF-04 (710bp), OPF-06 (380bp), OPG-12 (600bp), OPH-05 (740bp), OPI-18 (550bp), OPAB-01 (600bp), OPAE-07 (350bp), and OPQ-06 (200bp) showed a single band association with single trait out of eight , thought it were same or different, viz; PRX,OA, SOD, MDA content, and Injury index, proline content, MDA content, OA, SOD and Injury index respectively. 11 primers namely OPU-04 (650 &650bp), OPU-06 (1000&650bp), OPC-02 (440&410bp), OPAB-05 (300&675bp), OPP-07 (350&250bp) OPE-01 (950&900bp), OPI-08 (3000&750bp), OPI-14 (2000&550bp), OPB-05 (610&600Bp), OPR-07 (1200&480bp), and OPR-08 (950&430bp) exhibited two bands of different molecular bands were associated with different stress responsive traits (Table 23).

Three primers namely OPU-02 (510, 490, 410, 280, and 210bp), OPH-13 (830, 550 and 500bp) and OPAH-03 (1500, 950, 910, 780, 550, 260 and220bp) exhibited more than two bands associated with different traits. Primer OPU-02 exhibited 4 bands i.e., 510, 490, 410, 280, and 210bp were associated with different traits. Band 510 bp showed association with three traits namely osmotic adjustment, osmolality and proline content. Whereas bands 490,410,280 and 210bp showed association with only single trait i.e; P5CS, P5CS, osmolality and proline content respectively. Primer OPH-13 showed association of three bands having different molecular weight viz; 830, 550 and 500bp, band 830bp showed association with two traits i.e; OA and osmolality whereas band 550 and 500bp with osmolality and P5CS respectively. Primer AH-03, showed association with traits with maximum (7) bands of different molecular weights namely; 1500, 950, 910, 780, 550, 260 and220 bp. Out of these 7 bands 5 bands i.e; 1500, 950, 780, 550, and 220 showed the association with single trait (P5CS) only. Bands 910 and 260bp exhibited association with osmolality and Injury index respectively.

Table 23: RAPD bands and their association with stress responsive traits.

S.No	Primer	OA	Osmolality	MDA content	Proline content	Injury Index	PRX	SOD	P5CS
I	OPU-02-510bp	510**	510**	-	510**	-	-	-	-
	OPU-02-490bp	-	-	-	-	-	-	-	490**
	OPU-02-410 bp	-	-	-	-	-	-	-	410**
	OPU-02-280 bp	-	280***	-	-	-	-	-	-
	OPU-02-210 bp	-	-	-	210***	-	-		-
2	OPU-04 650 bp	650**	-	-	-	-	-	-	-
	OPU-04 590 bp	-	-	-	-	-	-	-	590**
3	OPU-06 1000kb	-	-	-	1000***	-	-	-	-
	OPU-06 650bp	650***	-	-	-	-	-	-	-
4	OPC-02 440 bp	-	440**	-	-	-	-	-	-
	OPC-02 410 bp	-	-	-	-	-	-	-	410**
5	OPAB-05 300 bp	300**	-	-	-	-	-	-	-
	OPAB-05 675 bp	-	-	-	-	-	-	-	675***
6	OPQ-09 570 bp	-	-	-	-	-	570**	-	-
7	OPR-04 400 bp	400**	-	-	-	-	-	-	-
8	OPR-06 850bp	-		-	-	-	-	850**	-
9	OPP-07 350 bp	-	-	-	350**	-	-	-	-
	OPP-07 250 bp	-	-	-	-	250**	-	-	-
10	OPE-01 950 bp	-	950**	-	-	-	-	-	-
	OPE-01 900 bp		900**	-	-	-	-	-	-
11	OPF-01 1000kb	-	-	1000**	-	-	-	-	-
12	OPF-04 710 bp	-	-	-	-	710**	-	-	-
13	OPF-06 380 bp	-	-		380**	-	-	-	-
14	OPG-12 600 bp	-	-	600**	-	--	-	-	-
15	OPH-05 740 bp	740**	-		-	-	-	-	-
16	OPH-13 830 bp	830***	830**	-	-	-	-	-	-
	OPH-13 550 bp	-	550**	-	-	-	-	-	-
	OPH-13 500 bp	-	500**	-	-	-	-	-	500**
17	OPI-08 3000bb	-	-	-	-	-	3000**	-	-
	OPI-08 750 bp	-	-	-	-	-	-	-	750**

18	OPI-14 2.0kb	-	-	-	2.0***	-	-	-	-
	OPI-14 550 bp	-	-	-	550**	-	-	-	-
19	OPI-18 550 bp	-		-	-	500***	-	-	-
20	OPAB-01 600 bp	600**	-	-	-	-	-	-	-
21	OPAE-07 350 bp	-	-	-	-	-	-	350**	-
22	OPAH-031500bb	-	-	-	-	-	-	-	1500**
	OPAH-03 950 bp	-	-	-	-	-	-	-	950**
	OPAH-03 910 bp	-	910**	-	-	-	-	-	-
	OPAH-03 780 bp	-	-	-	-	-	-	-	780***
	OPAH-03 550 bp	-	-	-	-	-	-	-	550***
	OPAH-03 260 bp	-	-	-	-	260**	-	-	-
	OPAH-03 220 bp	-	-	-	-	-	-	-	220**
23	OPB-05 610 bp	-	610**	-	-	-	-	-	-
	OPB-05 600 bp	-	-	-	600**	-	-	-	-
24	OPQ-06 200 bp	-	-	-	-	200**	-	-	-
25	OPR-07 1200bb	-	-	-	-	-	-	1200**	-
	OPR-07 480 bp	-	-	-	-	-	480**	-	-
26	OPR-08 950bp	-	-	-	-	-	950**	-	-
	OPR-08 430 bp	430**	-	-	-	-	-	-	-

** Significant at P= 0.01, *** Significant at P=0.001

Band size in base pairs (bp)

Association of STS marker:

In total 14 STS primers were used to develop DNA finger prints of 30 *Dichanthium* accessions. Out of these 7 primers revealed the association with one or more stress responsive traits. These primers namely 247P1/247P2, 284P1/284P2, 4P1/4P2, 15P1/15P1, T1F1/T1R1, AF1/AR1, and T3F3/T3R3 exhibited one or more bands of different molecular weight which showed association with one or more stress responsive traits (Table 24).

Out of 7 primers, 3 primers i.e., 4P1/4P2 (250bp), 15P1/15P1 (450bp), and T3F3/T3R3 (210bp) showed single band that was associated with proline content , osmolality, and proline content respectively. Three primers namely, 247P1/247P2 (300&200bp), 284P1/284P2 (680&430bP), AF1/AR1(360&220bp).Two bands in each primer indicated association with stress responsive traits, bands 300 &200bp of primer247P1/247P2 showed association with osmotic adjustment and P5CS enzyme activity respectively. Bands 680 and 430bbp of primer 284P1/284P2 were associated with P5CS enzyme activity and proline content respectively, whereas both (360&220bp) bands of primer AF1/AR1 were associated with single trait i.e., osmotic adjustment. Primer T1F1/T1R1 exhibited maximum (4) bands which showed association with one or more traits. Out of 4 bands three bands i.e., 800, 650 and 450 showed association with single trait (proline content), whereas band 430bp was associated with injury index (Table 24).

Association of ISSR marker:

Out of 5 ISSR primers, 4 primers namely ISSR 80, ISSR 81, ISSR 82, and ISSR 84 showed association with stress responsive traits. Two primers namely ISSR 81 and ISSR 84 exhibited single band association with single trait. Band 610 bp (ISSR 81) and 880bp (ISSR 84) showed association with P5CS enzyme activity and osmotic adjustment respectively. Whereas two bands from each ISSR 80 and ISSR 82 showed association with traits. Bands 710 and 425bp (ISSR 80) were shown association with MDA content only, whereas bands 400 and 250bp (ISSR 82) were associated with injury index (Table 25).

Table 24: STS bands and their bands association with stress responsive traits.

S. N0	Primer pair	OA	Osmolality	MDA content	Proline content	Injury Index	PRX	SOD	P5CS
1	SsCS247P1/SsCS247P2 200bp 300bp	200** -	- -	- -	- -	- -	- -	- -	- 300**
2	SsCS284P1/SsCS284P2 680bp 430bp	- -	- -	- -	- 430**	- -	- -	- -	680** -
3	SsCS4P1/SsCS4P2 250bp	-	-	-	250***	-	-	-	-
4	SsCS15P1/SsCS15P2 450bp	-	450**	-	-	-	-	-	-
5	SHST1F1/SHST1R1 1000bp 650bp 350bp 330bp	- - - -	- - - -	- - -	1000*** 650** 350***	- - - 330**	- - - -	- - - -	- - - -
6	SHCAPEAF1/SHCAPEAR1 360bp 220bp	360** 220**	360** 220*	360* -	- -	- -	- -	- -	- -
7	SHST3F3/SHST3R3 200bp	-	-	-	210**	-	-	-	-

** Significant at P= 0.01, *** Significant at P=0.001

Band size in base pairs (bp)

Table 25: ISSR bands and their association with stress responsive traits.

S. N0	Primer pair	Sequence	OA	Osmolality	MDA content	Proline content	Injury Index	PRX	SOD	P5CS	
1	ISSR 80	GC[CA]4									
	710bp	-	-	-	710**	-	-	-	-	-	-
	425bp	-	-	-	425**	-	-	-	-	-	-
2	ISSR 81	GT[CA]4									
	610bp		-	-	-	-	-	-	-	-	610**
3	ISSR 82	[AGAC]4GC									
	400bp		-	-	-	-	400**	-	-	-	-
	250bp		-	-	-	-	250**	-	-	-	-
4	ISSR 84	[GACA]4GT									
	880bp		880**	-	-	-	-	-	-	-	-

** Significant at P= 0.01, *** Significant at P= 0.001
Band size in base pairs (bp)

Discussion

Assessment of 30 *Dichanthium* accessions using RAPD marker system:

Introduction of molecular markers in plant breeding has provided a valuable tool for the characterization of genetic materials. Among them RAPD markers have been successfully used in *Dichanthium* (Chandra *et al.*, 2004; 2006) for germplasm evaluation because of their many advantages, however some doubts like reproducibility of RAPD bands have been expressed regarding the suitability of the RAPD markers for the genetic diversity study. The reproducibility of RAPD markers can be achieved by using optimized PCR condition especially high annealing temperature to reduce the minor bands (high stringency) and scoring only thick intense bands. In present study, similar strategy was use to get reproducible RAPD bands. The number of primers used in the method should be neither too small, because this could lead to non informative or biased analysis, nor too high which could result in increased cost. Chandra *et al.* (2006) used 32 random primers and detected 72.3% polymorphism in *Dichanthium* accessions. Joshi and Nguyen (1993) used 46 random primers in studying wild and cultivated wheat and detected 88% polymorphism. In the present study, 200 random primers were tested and finally 56 informative primers were chosen that amplified 3-19 polymorphic bands and revealed very high polymorphism (88.51%) in *Dichanthium* accessions. It is important to note that *Dichanthium* revealed significantly low amount of monomorphic bands (10.9%) in comparison to cultivated genotypes like lablab (31%) and rice varieties (33%) (Liu, 1996a; Ko *et al.*, 1994). This further supported the fact, that *Dichanthium* is a wild genus and ~~not much have~~ been domesticated. The efficiency of molecular marker technique depends upon the amount of the polymorphism so that it can discriminate among the set of accessions under investigation. In the present study RAPD not only generated a high number of polymorphic bands, but also the polymorphism, that was sufficiently distributed to enable discrimination among accessions by each primer. Consequently high (0.448) polymorphic information content (PIC) and

marker index (3.70) using RAPD marker system was obtained in 30 *Dichanthium* accessions. This further authenticated the utility and significance of this marker system in genetic relationship studies as supported by other studies like cashew (Archak *et al.*, 2003) and wheat accessions (Anna *et al.*, 2005).

Genetic variation and cluster analysis:

The cluster analysis (UPGMA) based on similarity matrix was performed to generate dendrogram. The distribution of 30 *Dichanthium* accessions revealed three (I,II and III) major clusters, which together constituted 29 accessions belonging to both distinct regions of the country (north India and south India).These accessions were clustered in high similarity range (72.61 to 95.36%). Accession IGBANG-D-2 (south India) separated from other clusters at very low similarity level (55.18%). Further, cluster II embodied maximum number of accessions (21) of which 20 accession belonged to North Indian central plateau and intermixing of accession IGKMFD-1 from South India. Accessions of this cluster (II) shared minimum 79.43% genetic similarity. Other two clusters (I and III) embodied 4 accessions in each, shared 84.76% and 79.43% genetic similarity respectively. Cluster II was separated from cluster I and II at 72.61 and 67.84% similarity level respectively, which indicated that cluster II was more genetically closer to cluster I than Cluster III. That was quite obvious also that all the accessions in this clusters (I) belonged to the north India. Accessions of Cluster III (south India) exhibited more genetic variability (79.43 to 86.84%) than the accession of the other clusters (I and II). However, the accession IGBANG-D-2 which separated individually from rest of accessions was closer to Cluster III. The study revealed considerable diversity among the accessions largely collected from two regions of India. RAPD marker system revealed only 9 accessions possessing more than 90% genetic similarity, rest of accessions exhibited similarity ranging from 55 to 90%. This wide range of genetic similarity among the accessions indicated a wide and diverse genetic base in the genus as earlier reported by Chandra *et al.* (2004). All six South Indian accessions showed wide variability

among themselves as well as to the accessions of north central India. Though these accessions were clustered in two different clusters but centered to one part of the dendrogram indicating some level of closeness making them to cluster close to each other. Similar results indicating a high level of diversity in isozyme banding pattern have been reported in guinea grass (Jain *et al.*, 2006), where authors emphasized evolution of new types through sexual recombination or other means namely hybridizing of indigenous materials with sexual/apomictic exotic lines. Guinea grass (*Panicum maximum* Jacq.) consists largely of apomictic population, although many sexual plants are also available in nature (Asseinan *et al.*, 1993). In case of *Dichanthium* though being largely apomictic in nature, the increased variability in sub-humid dry regions of south India might be due to the presence of major grasslands as well as favorable climate for natural crossing. Asseinan and Noirot (1995) have reported that apomixis does not lead to the reduction in the diversity of grasses. The *Dichanthium annulatum* complex, having different ploidy levels and growing wild in natural habitats, exhibits large phenotypic variation. Even diverse forms have been reported from material collected at the same locations and the clustering patterns based on agro-morphological attributes in marvel grass indicated independent groupings with their geographical distribution (Gupta and Gupta, 1993; Agarwal *et al.*, 1999). Genetic variation in *Dichanthium annulatum* genotypes by RAPD corroborated that diversity was independent of geographical distribution as genotypes from different areas clustered in the same group and vice versa (Chandra *et al.*, 2004). However, in present study when accessions of only two regions were analyzed most of south India accessions centered to one part of the phenogram as clusters possessing accessions of this region were close to each. In case of Napier grass (*Pennisetum purpureum*) both boot strap values and AMOVA analysis substantiated the results obtained from the dendrogram based on Dice similarity coefficients as that have not revealed any patterns *vis a vis* pedigree or source of collection (Bhandari *et al.*, 2006).

Assessment of ISSR marker system in 30 *Dichanthium* accessions:

ISSR technology is based on the amplification of region (100-3000 bp) between inversely oriented closely spaced microsatellite (Zietkiewicz *et al.*, 1994). Single primer 16-18 bp consisting of several simple sequences repeats used for amplification of these regions. ISSR usually amplify 25-50 products in one reaction, the number of bands produced may be negatively correlated with the number of nucleotides in the repeat unit of the motif as shown by Nagaraju *et al.* (2002) who investigate the genetic relationship between basmati and non-basmati rice verities. ISSR are also the most cost effective, as they can be resolved on agarose, no previous sequence information is required for design of the primers, and there is no need for using restriction enzymes. All these facts make them most attractive tool for analyzing genetic diversity and polymorphism among different accession. The major advantage of this method is the fact that it does not require a time consuming steps of genomic (or other) library construction. In spite of the fact that ISSRs are mostly inherited as dominant or rarely as co-dominant genetic marker. In the present study, ISSR marker system was used to develop DNA profile of 30 *Dichanthium* accessions belonging to two diverse regions of India. In total 61 amplified bands were scored (using 5 ISSR primers) with wide range of molecular weight (200bp to 2000bp).This marker system produced high polymorphism (71.42 to 100%) with average of 85.24% with *Dichanthium*. The polymorphic bands produced by these primers were ranged from 8-14 with average of 10.4 bands per primer. This high level of polymorphism clearly indicated the efficiency and utility of ISSR marker system in genetic relationship and trait association study in *Dichanthium,* supported by various studies especially in phylogenetic studies, the evaluation of genetic diversity and cultivars identification (Fang and Roose, 1994; Zietkiewicz *et al.*, 1994; Nagaraju *et al.*, 2002; Raina *et al.*, 2001). It was also observed in *Dichanthium*, that 10 nucleotides long primers with multiple di-nucleotide repeats were comparatively more polymorphic than long primers with four nucleotide repeats. Polymorphic information content (PIC) is characteristic of primer, which reflects the overall

suitability of marker system for the purpose of genotype identification, as it is related to the number of accessions distinguished by the primer (Gilbert *et al.*, 1999; Prevost *et al.*, 1999). High PIC value (0.405 to 0.50) with average of 0.48 among the 30 *Dichanthium* accessions was obtained. The marker index (MI) is the parameter specifically used for comparing the utility of two or marker systems (Powell *et al.*, 1996). High MI value (4.0 to 6.96) with high average 4.98 value using ISSR marker system for discrimination of *Dichanthium* accessions make it useful tool. ISSR is a derivative marker system derived from SSR hence this PCR based system produce a number of polymorphic bands for each PCR reaction and exhibit high polymorphic information content (PIC) values (Blair *et al.*, 1999).

Genetic variation and cluster Analysis:

Wide genetic diversity was obtained among the *Dichanthium* accessions using ISSR marker system. Dendrogram revealed 3 major clusters (I, II and III) which together constituted 28 *Dichanthium* accessions. Two accessions (IGBANG-D-2 and IG97-144) distinctly separated from rest of accessions and did not part in any of the cluster and joined rest of the accessions at 62.21 % and 56.77 % of genetic similarity respectively. Genetic similarity revealed by dendrogram among accessions ranged from 66.6% to 100%. Cluster II embodied 17 accessions shared more than 72% similarity. However 2 pairs of accessions in this cluster shared 100% similarity, whereas rest of the accessions in the cluster showed wide variations and polymorphism. The intermixing of IGKMFD-1 (South India) which shared 73.76 % similarity with the other accessions of cluster II was noticed. Total 5 notes were obtained in dendrogram possessed more than 50% boot strap value. A group of 4 accessions (IG97-234, IG95-30, IG97-152 and IG97-184) belonging to North India central plateau showed a high genetic similarity (81.08%) as indicated by high boot strap (82.5) value. Intra-cluster similarity in Cluster I ranged from 81.08 to 100 % which indicated that these accessions are very closely related. Cluster III embodied 7 accessions (IG97-121, IG97-244, IG97-245, IGTGD-4, IGKMD-10, IG3108 and IGBANG-D-1) where 4

accessions belonging to south India and 3 accessions (IG97-121, IG97-244, IG97-245) belonging to north India shared (100%) genetic similarity indicated close genetic relationship to the accession of south India. This close genetic relationship further indicated their wide and well distributed genetic base despite their different regional distribution. The genetic variations in *Dichanthium* accessions obtained using ISSR marker system corroborated that largely accessions were more or less separated on the regional basis. Though 3 accessions from south India clustered with accessions of south India, yet they were close to north Indian accessions in dendrogram. Further Cluster III was separated from Cluster-II and Cluster I at 68.43% and 66.6% genetic similarity level respectively. All the accessions belonging to cluster III were grouped at minimum 78.46% genetic similarity. In total 3 notes were obtained having more than 50 % boot strap value in this cluster (III).

The boot strap value ranged from 7.1 to 100% at various nodes of dendrogram indicated better clustering significance. Total 12 nodes in dendrogram were identified, which exhibited more than 50% boot strap value. The genotype with boot strap value below 50 % indicated that the position of these accessions may change if other marker system or other accessions are included in the analysis.

Simplicity of ISSR markers predetermines them for gene tagging; as reported in wheat where association of ISSR with seed size was tested (AmmiRaju *et al.*, 2001), gene tagging by mean of ISSR for identification of a tight linkage between a marker and nuclear restores gene in rice (Nagaraju 2002). ISSR markers are highly useful for monitoring somaclonal variations (Albani *et al.*, 1998). Evaluation of genetic diversity using ISSR have been carried out in various species i.e., common bean (Galavan *et al.*, 2003), peanut (Raina et *al.*, 2001) and citrus (Fang and Roose, 1994) showed easiness associated with this marker system.

Assessment 30 *Dichanthium* accessions using STS marker system:

Seventeen sequence-tagged-sites (STS) primers (reverse and forward primer) were used to develop DNA finger prints of *Dichanthium* accessions. Since these primers were designed from a fodder legume (*Stylosanthes*), hence the transferability of STS markers in *Dichanthium* was tested. STS primers were allowed for amplification reactions at three different annealing temperatures (52°C, 55°C and 58°C). Out of 17 STS primers, 14 performed well and gave amplification reactions at 52°C. STS markers have proved to be extremely valuable tool in the analysis of gene pool variation of crops during the process of cultivar development, and classification of germplasm. These markers are extremely sensitive and can detect allelic variability during cultivar development. Among *Dichanthium* accessions this marker system successfully dissecting the genetic diversity and revealed high polymorphism (80.1%) ranged from 66.66 to 100%. In total 106 bands were amplified ranged from 4 to11 bands with average of 7.57 band per primer. high polymorphism in *Dichanthium* accessions was supported by various studies in different plants i.e., *Lolium*. Patricia and Lallemand (2003) developed STS markers from *Lolium* sequences especially using consensus sequences from related species of *Gramineae*. The primer pairs were designed in order to amplify the intronic regions thus, the polymorphism detected was based on intronic length polymorphism. Out of 42 STS markers, 85.8% yielded successful amplification and 62% revealed a high level of polymorphism. The analysis of amplicons revealed a high STS marker specificity, more over, the majority of the STS markers can be considered as "universal markers" because 81% of these STS markers amplified successfully across 20 related grass species. However, fodder grasses have been less intensively studied than other members of *Poaceae* and few DNA sequences are developed. Suitability and utility of STS marker system in estimating genetic diversity in *Dichanthium* accessions was further evaluated by estimating the polymorphic information content (PIC) and marker index, a fairly high PIC values were obtained ranged from 0.222 to 0.499 with an average of 0.426. Whereas MI

values obtained ranged from 1.33 to 4.99 with an average of 2.60 was sufficient enough to compare the utility of two or marker systems (Powell *et al.*, 1996)

Genetic variation and cluster analysis:

Dendrogram generated based on STS marker revealed the 3 major clusters namely cluster I, cluster II and cluster III. These clusters were separated at 75% genetic similarity level. Out of 30, 29 accessions were grouped in various clusters except one accession i.e., IGBANG-D-2. Accession (IGBANG-D-2) was belonging to south India and separated from rest of the accessions at 70% genetic similarity level (Figure 7).Out of 30 accessions 9 accessions all belonging to North India, shown 100% similarity could not be distinguished using this marker system, rest of 21 accessions showed genetic similarity ranged from 70 to 98 %. Dendrogram and clustering pattern using this marker system fairly separated accessions on the basis of their regional distribution, 5 out of 6 accessions from south India were clustered together in the similarity range of 80 to 84%. Intermixing of accession IG97-144 from north India indicated its close similarity with south Indian accessions. Total 12 nodes in dendrogram were identified, which exhibited more than 50% bootstrap value. The genotype with boot strap value below 50 % indicated that the position of these accessions may change if other marker system or other accessions are included in the analysis.

Assessment of isozyme marker system in 30 *Dichanthium* accessions:

A total of 16 isozyme loci with 55 alleles were detected using four enzyme systems in 30 accessions of *Dicharthium*. The percentage of polymorphic loci ranged from 37.50 to 75(IG97-234) accession wise. Accessions IG97-152, IG97-130, IG97-121, IG97-244, IG97-245 and IG97-144 depicted least (37.75%) polymorphism where 6 out of 16 loci were polymorphic. Maximum polymorphism (75%) observed in accession IG97-234 where 12 out of 16 loci were polymorphic. The number of polymorphic loci in other accessions ranged

from 7 to 11 out of 16 loci with polymorphism ranging from 43.75 to 68.87. Out of 30 accessions 8 was detected possessing 50 % polymorphic loci where 8 out of 16 loci were polymorphic, the second highest (68.87%) polymorphic loci were observed in 4 accessions where 11 out of 16 loci were polymorphic followed by 6 accessions having 56.25 polymorphic loci. The number of accessions having 50% polymorphism was maximum (eight) followed by 43.75 and 37.75 each having six accessions. The number of monomorphic loci also varied among the accessions. The maximum number of such loci was 10 observed in six accessions and minimum was 4 and observed in only one genotype IG97-234.

The allelic frequencies in 30 accessions of *Dichanthium* were calculated from 16 loci with 55 alleles. The 16 enzyme coding loci coded by 4 enzyme systems, esterase was the most polymorphic enzyme system produced 22 alleles of which 20 was polymorphic. Locus 4 of esterase showing 7 alleles, 29 accessions out of 30 were polymorphic which accounts 96.6% of polymorphism to this locus. The second most polymorphic locus was PPO3 having 4 alleles polymorphic and 90% of accessions were polymorphic to this locus. PRX1 locus observed as the least polymorphic with only 16.6% of accessions were polymorphic out of 30 accessions, for rest of the polymorphic loci, 23.3 to 83.3 % of accessions were observed polymorphic.

Mean heterozygosity among the polymorphic loci in *Dichanthium* germplasm studied presently was comparatively high (0.406-0.836) to that reported in crops like red clover, sunflower and guar (Carrera and Poverene, 1995; Kongkiatngam *et al.*, 1995; Ebrahmi *et al.*, 2004). However, Napier grass where three enzyme systems reported to identify more than 90 % accessions indicated a high level of genetic diversity (Bhandari *et al.*, 2006). Isozyme analysis in *Cenchrus,* a tropical grass also depicted high level of polymorphism (Dubey, 2004). Of 16 isozyme loci observed in present work, EST1 with maximum heterozygosity (0.836) indicated the importance of a particular locus in determining the genetic diversity in *Dichanthium.* Similarly, at accession level, maximum heterozygosity (0.743) was observed in IGKMD-10. Other three

accessions viz., IGKMFD-1, IGTGD-4, IGBANGD-2 of the same region (south India), depicted heterozygosity more than 0.70.An average PIC value of 0.46 across all scored RAPD bands, as well as an average MI of 4.34 across all primers obtained with *Dichanthium* accessions were different than that of AFLP-based genetic diversity studies in various crops (Powell *et al.*, 1996; Muminovic *et al.*, 2004).

Genetic variation and cluster analysis

The study revealed considerable diversity among the genotypes collected from two regions of India. Due to less discriminatory power of isozyme marker system, 18 accessions out of 30 shared more than 90% similarity whereas rest of the 12 exhibited similarity ranging from 67 to 90%. All 18 accessions were from central north India. Four enzymes system showed 100 % similarity among six of these 18 accessions. Out of twelve genotypes which have shown similarity of the level of 67 to 90%, six accessions were from south India exhibited higher level of diversity among themselves. The closest two accessions of this region was IG3108 and IGBANG-D-1 having 88 % similarity. Clustering patterns based on isozyme banding patterns clearly indicated that accessions of south India clustered together though having higher level of diversity among themselves, four accessions belonging to central north India possessing 100 % similarity intermixed with the members of south India. In cluster-1 all accessions observed were of the same region or closely located along with accessions having 100 % similarity, however in cluster-3 accessions were of contrasting regions with that of accessions having 100 % similarity, indicated presence of diverse accessions in cluster-3. All six accessions belonging to south India were highly divergent and two nearest accessions in this category were IG3108 and IGBANG-D-1. Though the four north central Indian genotypes showing 100 % homology clustered with two south Indian genotypes, the rest of the genotypes belonging to two different zones showed distinct level of polymorphism. If the result of the phenogram is used to explain the divergence of the genotypes from common ancestors, it seems

that clusters-1 and 2 diverged at nearly same level. In cluster 1, sub-cluster 1-1 of five accessions diverged earlier than that of sub-cluster 1-2. In cluster-2 the sub-cluster 2-3 incorporating three accessions of south Indian region diverged early followed by sub-cluster 2-2 having one south Indian accession. Cluster-3 diverged late in comparison to other two clusters but in this cluster IGBANG-D-2 genotype of south India diverged earlier than rest of the genotypes. All six south Indian accessions analyzed showed wide variability among themselves as well as to the accessions of north central India. Though these accessions were clustered in two different clusters but centered to one part of the phenogram indicated some levels of closeness making them to cluster close to each other. A high level of diversity in isozyme banding patterns have been reported in guinea grass (Jain *et al.*, 2006), where authors emphasized evolution of new types through sexual recombination or other means namely hybridizing of indigenous materials with sexual/apomictic exotic lines. The *Dichanthium annulatum* complex, having different ploidy levels and growing wild in natural habitats, exhibits large phenotypic variation. Even diverse forms have been reported from material collected at the same locations and the clustering patterns based on agro-morphological attributes in marvel grass indicated independent groupings with their geographical distribution (Gupta and Gupta, 1993; Agarwal *et al.*, 1999). However, in present study when accessions of only two contrasting regions were analyzed, most of the accessions of south India centered to one part of the phenogram.

Comparison of RAPD, STS, ISSR and isozyme marker systems in genetic diversity assessment of 30 *Dichanthium* accessions:

In the present study an attempt was made to compare the utility of four markers system viz., ISSR, RAPD, STS, and isozyme in studying genetic diversity of 30 *Dichanthium* accessions. The performance of these markers system were evaluated using various parameters, such as present of polymorphism, polymorphic information content (PIC) marker index (MI) and clusters formed in dendrogram. The results indicated that mean number of bands per primer (assay)

yielded (9.1) RAPD (9.1), (12.2) ISSR, (7.57) STS and (13.75) in isozyme (Table 26). It was higher in isozyme but among PCR based DNA marker systems, highest number of bands per primer was generated by ISSR followed by RAPD and least in STS marker system. More over the mean number of polymorphic bands par assay indicated that maximum number of polymorphic bands par assay was obtained in ISSR (10.4) followed by RAPD (8.10) and minimum in STS (6.0), whereas isozyme generated 11.0 bands par assay unit. However, the percentage of polymorphism ranged from 88.51 to 74.51%, where maximum polymorphism was detected in RAPD (88.51%) followed by ISSR (85.24%), and 80.10% and 74.51% polymorphism was detected in STS and isozyme marker systems respectively. Based on polymorphism it was suggested that all four marker systems are suitable for discrimination of *Dichanthium* accessions and assessment of their genetic diversity. However STS and isozymes are comparatively less efficient in genetic diversity estimate of *Dichanthium* accessions as compared with ISSR and RAPD marker systems. Mean polymorphic information content (PIC) par assay (0.480) in ISSR was highest among the marker systems used, followed by (0.448) in RAPD, (0.426) in STS and minimum (0.419) in isozyme marker system. The marker index parameters have been specially used for comparing the utility of two or more marker systems (Powell et al., 1996). Although the isozyme system indicated higher (5.25) value of MI but less polymorphism does not make this system most discriminative. Among the DNA based molecular markers ISSR generated higher MI (4.98) value than RAPD (3.72) and STS (2.60). However the results in the study suggested that all the marker systems examined turned out to be a useful tool for detection of polymorphism and assessing the genetic diversity in *Dichanthium* genotypes. Although the PIC and MI values of STS marker system were relatively low in comparison to other (ISSR, RAPD and isozyme) marker systems applied in *Dichanthium*. It has advantage of being locus specific, which is valuable in some genetic investigations. The similar results were found in winter rye (Hanna *et al.*, 2005). Though the very less numbers (5) of ISSR primers were used in present

Table 26: Comparative analysis of banding patterns generated by ISSR, RAPD, STS, and isozyme markers system for 30 *Dichanthium* accessions.

S.No	Components	ISSR	RAPD	STS	Isozyme
1	No of assay units	5	56	14	4
2	Total no of bands	61	510	106	55
3	Mean no of bands/ per assay unit	12.2	9.10	7.57	13.75
4	Total no of polymorphic bands	52	454	84	44
5	No of polymorphic bands par assay unit	10.4	8.10	6.0	11.0
6	Mean (%) polymorphism par assay	85.24	88.51	80.10	74.51
7	No of monomorphic bands par assay	1.8	1.0	1.57	2.75
8	Mean PIC par assay	0.480	0.448	0.426	0.419
9	Mean Marker Index (MI)	4.98	3.72	2.60	5.25

study, however they turned out to be very effective in detecting polymorphism in *Dichanthium* accessions .

Comparison of dendrogram and clustering pattern and grouping of *Dichanthium* accessions obtained from RAPD, ISSR, STS and isozyme marker systems:

The dendrogram obtained form these 4 markers systems revealed 3 major clusters which together constituted 29 (RAPD), 28 (ISSR), 29 (STS) and 30 accessions in isozyme based markers. The minimum genetic similarity among 4 marker systems in *Dichanthium* accessions ranged from 55 to 70%. Low similarity was detected with RAPD (55%) and ISSR (56.7%). Hence, the genetic variation detected among *Dichanthium* accessions by RAPD and ISSR were higher than those of STS and isozyme. Bhattin (2003) assayed 15 wild barley populations from west Turkey by using RAPD and ISSR markers and obtained (0.27) average genetic similarity, hence genetic variation obtained using RAPD and ISSR marker system was higher than those reported with isozyme by Nevo *et al.* (1979) and Nevo *et al.* (1986). Though all four marker system in the present study revealed 3 major clusters in the dendrogram, but the distribution of accessions in the clusters were not identical in all the cases and hence certain degree of variations among the accessions forming the cluster was persist. However certain patterns of grouping revealed by cluster analyses were consistent across dendrograms obtained using data from each marker system i.e., accession pairs IG 97-234 and IG95-30, IG97-152 and IG97184 were always appeared together in dendrograms of all marker systems especially in cluster I, which invariably clustered with accessions belonging to the North India. Thought the accession pair IG97-152 and IG97184 in case of isozyme appeared in cluster II but it was very close to cluster I. High boot strap value (>50 %) further indicated their strong association. Another set of four accessions (IG97-121, IG97-1244, IG97-245 and IG97-144) appeared together in all dendrograms, however their position in clusters were varied from cluster II (in RAPD and STS) to Cluster III (in ISSR and Isozyme) though

accession IG97-144 were separated from its group but it was still closer to these accessions. Another accessions of pairs (IG97-147 & IG97-170, IG97-118& IG97-233, IG97-189 & IG97-132) also appeared together in cluster II of all dendrograms. Distribution of accessions based on their geographical distribution in country obtained from using different marker system in their corresponding dendrograms and clustering patterns were more or less accorded with geographical distribution, however intermixing of one or two accessions as in case of RAPD and ISSR accession IGKMFD-1 and in case of isozyme (IGKMD-10 and IGBAND-D-2) with North Indian accessions was visualized. Accession (IGBANG-D-2) separated as single accession in RAPD, ISSR, STS, though this accession was closer the south Indian accessions. Rest of four south Indian accessions (IGTGD-4, IGBANG-D-1, IGKMD-10, and IG3108) clustered together.

When results of all 4 markers systems was taken together some of the accessions namely IGKMFD-1, IGTGD-4 and IGBANG-D-2 of south India and genotypes from the group of IG97-189, IG97-132 as well as IG97-121, IG97-244, IG97-245 and IG97-144 of central north India were identified as distinct as they have depicted high level of variations and unique clustering pattern to that of rest of the accessions. These three south Indian accessions either got intermixed with the rest of the north Indian genotypes or formed separate cluster than that of the rest of the genotypes of the same region. The accessions of north India though have shown high level of similarity among themselves but were either in between the south Indian clusters or highly dissimilar to the rest of the accessions of the same region. In case of RAPD, IGBANG-D-2 accession of south India showed only 45 % similarity with the rest of the genotypes whereas in case of ISSR (62.21%), STS (70%) and in case of isozyme it has shown and 67 % similarity with the rest of the clusters. Both marker systems showed that genotype IGKMFD-1 of south India was closest to the central north Indian accessions. The same genotype got intermixed with other accessions of north India as revealed by RAPD indicated better diversity in this genotype in comparison to other

genotypes of this region. The study indicated that ISSR and RAPD marker system have a clear cut edge over STS and isozyme marker system.

Stress studies

Relative water content (RWC):

The metabolic activity in leaf tissues can be evaluated by measuring RWC that can be considered as integrated measure of plant water status (Flower and Ludlow, 1986). In cereals it has been demonstrated that resistance to drought stress is a quantitative trait and that RWC is relevant tool for screening drought tolerance (Teulate *et al.,* 2003). In fact, RWC gives an idea at a specific point of time the level of water deficit and this has been used as a first hand information about response to drought, in the present study. All accessions were subjected to water stress by with holding water, and aim was to brought down the RWC to the level of 53-43%. Seven accessions (IG97-234, IG97-24, IG95-30, IGKMFD-1, IG97-247, IG97-152, and IG97-180) took maximum (11-14) days to come to the level of 47%, 5 accession namely IGTGD-4, IGBANG-D-2, IGKMD-10, IG 3108, and IGBANG-D-1 took less days (6-7) to come to the level of 55-46 % while rest of the accession took 8-10 days to come to this level. The results indicated that those accessions took maximum days to drop their RWC to minimum level than those accessions which took less number of days may have better ability to sustain water stress and the accessions which drop their RWC early may be sensitive toward water stress and remaining accessions were considered in moderate category.

Water potential (WP):

The most common parameters used to assess the severity of drought are leaf water potential and relative water content (Cornic, 1994; Lower, 1995). In general increase of water stress causes decrease in water potential (more negative). The present study indicated that leaf water potential decreased uniformly as magnitude of water stress increased. But that differ in extent of decrease accession wise.

Based on the decrease in water potential under water stress, 30 Dichanthium accessions were put into three probable categories. Four accessions (IG97-24, IG97-184, IG97-218, and IGBANG-D-2) showed maximum decrease (more negative) in water potential ranged from -5.0 to -6.02 MPa. Five accessions (IG97-158, IG97-118, IGTGD-4, IGKMD-10 and IG3108) showed least decrease in water potential (less negative value) ranged from –2.56 to –2.93MPa. Other accessions showed moderate decrease in water potential (-3.0 to - 4.9MPa) under severe water stress condition. However as far as difference in water potential (ΔWP) under control and stress condition was concerned, a group of 7 accessions (IG97-24, IGBANG-D-2, IG97-151, IG97-121, IG95- 30, IG97-114 and IG97-130) showed maximum difference in WP (ΔWP) (-3.0 to - 4.57 MPa) from their respective control and a group of 4 accessions (IG97-158, IG3108, IG97-147 and IGKMD-10) showed least difference (less decrease) in WP (ΔWP) from their control under water stress conditions, however, their RWC was maintained at lower value (46.83%). Since the two groups of accession revealed significant difference in their WP (ΔWP), further indicated the greater ability of accumulation of solutes in those accessions where maximum decrease (more negative) and difference in WP from control was observed, than those of showing less decrease (less Negative) in WP. Similar results were obtained (Moinuddin et al., 2004) in chick pea where contrasting HOA (tolerant) and LOA (susceptible) chick pea cultivars were studied for their water relation parameters where HOA cultivars exhibited lowest WP (more negative) than those of LOA cultivars. Morgan (1995) also obtained similar results with wheat lines differing in osmoregulatory capacity.

Water loss rate (WLR):

The dynamics of leaf water loss was measured by WLR test on 30 *Dichanthium* accessions. A group of 9 accessions namely IG97-234, IG97-24, IG95-30, IG97-241, IG95-25, IG97-147, IG97-170, IG97-130, IG97-144 indicated least water loss accounts for only 35-42% where as maximum water loss was observed in

IG3108, IGTGD-4 and IG97-118) accessions which accounts for 52-54% or more loss. Rest of the accessions were in between these two extremes. The difference of water loss (WLR) after 2 hrs differed significantly between the accessions of less WLR (tolerant) and High WLR (susceptible) but the difference in WLR reduced as time progressed and become insignificant after 24 hrs. Those accessions registered less water loss in 2 hrs were considered as drought tolerant, and those which register maximum water loss as drought susceptible and rest were as moderate. Similar results have been reported in wheat (Patrizia *et al.*, 2006) depicting the role of WLR in screening of lines at least at preliminary level.

Proline content:

Accumulation of high proline content in cells has been associated with preventions of protein denaturations (Kumar *et al.*,1994) prevention of enzyme structure and activity (Samuel *et al.*, 2000), and protection of membranes from damage by ROS, produced under drought and high light conditions (Saradhi *et al.*, 1995). Proline content in *Dichanthium* observed many fold increase under water stress, however its extent was different in accessions. Six accessions (IGBANG-D-2, IG97-118, IG97-147, IGKMFD-1, IGKMD-10 and IG95-30) showed high increase (108.6 to 163.8 fold) over their respective controls. These accessions also maintained RWC level from 53-41%. Contrary to this, a group of 3 accessions (IG97-170, IG97-241 and IG97-121) accumulated less 25-30% fold proline content over their control having water stress to the level of 51% RWC indicating variations in accumulation of proline under stress (similar level of RWC) by different accessions. The opinions reported on the importance of proline for measuring stress responses are still contradictory (Hare and Cress, 1997). For many plant and animals species proline accumulation during stress is an established fact that can be considered an indicative response to stress at the cellular level. However, to date the exact mechanism of its action in the adaptation to stress has not yet completely elucidated. Free proline might be involved in membrane stabilization during water stress (Kocheva and Georgiev,

(2003) or it might be a reserve of readily mobilizable N, available upon relief to stress (Hare *et al.*, 1998). The value of free proline content also appeared to be related to resistant or sensitive characteristic toward stress. In the present study, proline increase was parallel to RWC decrease among the genotypes recorded, however the extent of proline increased was variable among the accessions, it is therefore the accessions indicated higher accumulation of proline when RWC was low may have better capability to sustain water stress and could turned to be tolerant kind, this was accordance with data reported for other species (Sudershan and Sudhakaran, 1995; Ain-Lohut *et al.*, 2001; Peuke *et al.*, 2002). Nevertheless the data reported on the role of proline accumulation in conferring tolerance to drought stress are highly controversial and it is still not clear whether or not the increase in proline levels can counter enhanced drought tolerance (De-Ronde *et al.*, 2000; Nayyar and Walia, 2003.)

Osmotic potential:

The values of osmotic potential (OP) are generally less negative at controlled conditions however when water stress was subjected to plants, osmotic potential (OP) further declined and become more negative (decrease). However, the extent of decrease in OP varies among the accessions in accordance with water potential (WP) and relatives water content (RWC). The present study indicated that there was a significant difference in osmotic potential (OP) under control and stressed plants. Highest decrease (more negative) in OP (-3.44 to -4.15 MPa) was observed in a group of accessions (IG97-234, IG97-24, IG95-114, IG97-151, IG97-247 and IG97-241). Similarly the higher difference in OP (ΔOP) (from control to stress) among the group of accessions (IG97-242, IG97-234, IG97-241, IG95-114, IGKMFD-125, IGBANG-D-2) ranged (-2.10 to –2.92 MPa) under water stress. The lowest decrease (less negative OP) was recorded in a group of accession (IG97-121, IG97-244, IG97-245, IG97-144 and IGTGD-4) ranged from (-2.5 to –2.10 MPa). Concurrently less difference in OP (ΔOP) form their respective control was recorded in accessions (IG97-121, IG97-244, IG97-245

IG3108 and IGBANG-D-1). Rest of the accession moderately decreased (more negative) their OP under water stress. Accession having high OP are considered as better equipped to deal the water stress and these accession were also capable of maintaining higher RWC. The maximum difference in OP (ΔOP) from control to stress is good indicator for accessions having better capability for dealing water stress (tolerant type), in contrast to those accessions which are not capable of decreasing the osmotic potential (less negative value) and difference in (ΔOP) (less negative value) from control to stress.

Osmotic adjustment:

Osmotic adjustment is considered as a physiological mechanism of drought adaptation in many crop plants. Osmotic adjustment (OA) capacity of Dichanthium accession was compared among the accessions under water stress. Out of 30 accessions, 3 accessions (IG97-130, IG 97-121 and IG97-244) where osmotic adjustment (OA) values were in negative. Rest of the 27, nine accessions (IG97-24, IGKMFD-1, IGBANG-D-2, IG97-114, IG97-234, IGTGD-4, IG95-30, IG97-233, and IG97-241) have indicated high osmotic adjustment and such accessions can be put into high OA group and they were better capable of tolerating water stress. A group of 10 accessions (IGBANG-D-1, IG3108, IG97-144, IG97-245, IG97-218, IG97-132, IG97-189, IG97-152, IG95-151, IG97-147) indicated low OA values (0.05 to 0.163) can be regarded as low OA group because of their low osmotic adjustment capacity. Rests were put into moderate group. Both high OA group and low OA groups showed decrease (more negative) in OP_{100} with decrease in WP at water stress condition. Further OP_{100} (osmotic potential at full turgor in stress) was also more negative (lower) in those accession which indicated higher osmotic adjustment capacity. Accessions of high OA namely IG97-24, IGKMFD-1, IGBANG-D-2, IG97-114, IG97-234, IGTGD-4, IG95-30, IG97-233, and IG97-24 showed average OP_{100} (-1.96MPa) and accession of low OA showed average OP_{100} (-1.56 MPa), whereas in rest of the accessions OP_{100} were moderately decreased and consequently moderate osmotic adjustment (OA) capacity was seen. Moinuddin et al. (2004) in chick pea Similar results were also by

cultivars and Kumar and Singh (1998) in Brassica species also observed similar results. Francisco et al. (1998) working on pea cultivars supported the fact that the cultivars which have best maintained the turgor were those which were more drought tolerance. This indicated that the turgor maintenance was significantly related to osmotic adjustment. So OA can also be used as screening technique for drought tolerance (Kumar and Singh, 1998). Hence the results of present study indicated that those Dichanthium accessions maintained higher OP_{100}, WP and consequently osmotic adjustment (OA) can be defined as drought tolerant accessions.

MDA content:

The drought induced changes in the level of lipid peroxidation in terms of MDA content can be attributed to sort out accessions for their tolerance toward water stress. Results indicated that a significant difference in MDA content under stress condition was obtained in most of the accessions. Out of 30 accessions, 9 namely IGKMFD-1, IG 97-152, IG 97-241, IG97-158, IG95-114, IG97-24, IG95-25, IG95-30 and IG 97-121 showed high increase in MDA content over their respective controls (30 to 68%) (Table 27), whereas 7 accessions (IG97-144, IG97-189, IG97-244, IGTGD-4, IGBANG-D-2, IG97-192 and IG97-247) exhibited less MDA content (4.78% - 21.27%) (Table 28). Rest of the accessions showed moderate increase in MDA content. Out of 30, six accessions did not show a significance difference in MDA content at control and stress condition. The different rate in increase of MDA content has been reported in two species of jute resulted the difference in membrane permeability as a result of increased lipid peroxidation (Chowdhury and Chowdhaury, 1985). Sudden increase of MDA with water stress caused higher lipid peroxidation and thus the species would be more vulnerable (susceptible) to stress. The level of MDA sharply increased with increased magnitude of water stress (Dhindsa and Motowe, 1981). The MDA content is expressed on protein basis because the greater part of the lipids in wheat leaves is in the form of membranes, particularly those which are associated with chloroplasts and chloroplasts proteins (Price and Hendry, 1991). The

Table 27: Putative drought tolerant accessions categorized with individual parameter.

So No	Accessions	Probable category	Parameters	Remarks
1	IG97-234, IG97-24, IG95-30, IG97-247, IG97-152, IG97-184, IGKMFD-1.	Drought tolerant	RWC	11-13 days
2	IG97-234, IG97-24, IG95-30, IG97-241, IG95-25, IG97-147, IG97-132, IG97-130, IG97-144.	Drought tolerant	WLR	Low
3	IG97-234, IG97-24, IG97-247, IG97-241, IG95-114, IG97-151.	Drought tolerant	OP	Decreased
4	IG97-24, IG97-184, IG97-218, IGBANG-D-2.	Drought tolerant	WP	Decreased
5	IG95-30, IGKMFD-1, IG97-241 IGBANG-D-2, IG95-114.	Drought tolerant	osmolality	Increased
6	IGBANG-D-2, IG97-147, IGKMFD-1, IGKMD-10, IG97-130, IG97-118.	Drought tolerant	proline	Increased
7	IG97-234, IG97-24, IG95-30, IG97-192, IG97-247, IG97-241, IGBANG-D-2, IGKMD-10, IGBANG-D-1.	Drought tolerant	Injury Index	less
8	IG97-234, IG97-24, IG95-30, IGKMFD-1, IG97-241, IGBANG-D-2, IG95-114, IG97-233, IGTGD-4.	Drought tolerant	OA	High
9	IG97-234, IG97-247, IG97-192, IG97-189, IG97-244, IG97-144, IGTGD-4.	Drought tolerant	MDA	Low
10	IGKMFD-1, IG97-241, IG97-147, IG97-158, IG97-130, IGTGD-4, IGBANG-D-1.	Drought tolerant	P5CS	Increased (more)
11	IGBANG-D-2, IGTGD-4, IGKMD-10, IG97-118, IG97-170.	Drought tolerant	POD	increased
12	IG97-24, IG97-247, IG95-25, IG97-170, IG97-158, IG97-132, IG97-18, IG97-130, IGBANG-D-2, IGKMD-10.	Drought tolerant	SOD	Decreased

Dichanthium accessions which indicated high level of MDA content, indicated more lipid peroxidation and more membrane permeability, are comparatively more susceptible for water stress than those which produce less MDA content at higher magnitude of water stress, such accessions have better capability for water stress tolerance. The decreased lipid peroxidation (low MDA content) has been attributed to the expression of genes, Glutathione synthetase and HAL2 (sulphur metabolism) for increase in glutathione, as indicator of oxidations stress resistance in transgenic lines of tobacco (Singh and Verma, 2001). Similarly the selected lines of rice showed less accumulation of MDA and were found superior to other lines in terms of water stress tolerance (Reddy *et al.*, 1988).

Injury Index:

Membrane injury as a consequence of water stress can be used as a tool for screening of drought/salinity tolerance. In the present study, data obtained regarding the membrane injury (as injury index) indicated that extent of injury under moisture stress condition was variable among the 30 *Dichanthium* genotypes. Maximum membrane damage resulting injury index (I) was observed in accessions IGTGD-4 (23.63%) and IG 3108 (20.0%). Beside these, 8 other accessions namely IG97-147, IG97-118, IG97-132, IG97-144, IG97-151, IG97-152, IG97-158,and IGKMFD-1 indicated higher injury index (13.19 to23.63%) where as 9 accessions (IG97-234, IG97-24, IG95-30, IG97-192, IG97-247, IG97-241,IGBANG-D-2,IGKMD-10 and IGBANG-D-1) indicated less injury index (4.78 to8.54%) with average of 6.16% under the severe water stress condition. Rest of the accessions showed moderate membrane injury, hence injury index (I) (8.6 to13.9%). Several authors reported that injury index corresponds with magnitude of water stress (Blum and Ebercon, 1981; Krishnamani *et al.*, 1984). According to Blum and Ebercon (1981) injury index signifies the degree of membrane damage as inflicted by water stress. In the present study the higher injury index as revealed by 10 accession especially IGTGD-4 and G 3108 (23.63 and 20% respectively) at average RWC (49%) indicated that these accessions are

Table 28: Putative susceptible accessions categorized with individual parameter.

So No	Accessions	Probable category	Parameters	Remarks
1	IG3108, IG97-24, IGBANG-D-2, IGKMD-10, IBANG-D-1.	Drought susceptible	RWC	6-8 days
2	IG3108, IG97-118.	Drought susceptible	WLR	More
3	IG3108, IG97-121 IG97-244, IG97-245, IG97-144, IGTGD-4, IBANG-D-1.	Drought susceptible	OP	more
4	IG3108, IGTGD-4, IGKMD-10, IG97-158, IG97-118.	Drought susceptible	WP	more
5	IG3108, IG97-121 IG97-245, IBANG-D-1, IG97-130.	Drought susceptible	osmolality	Low
6	IG3108, IG97-121, IGTGD-4, IG97-192, IG97-247, IG97-241, IG97-170.	Drought susceptible	proline	Low
7	IG3108, IG97-144, IG97-118, IG97-152, IG97-151, IGTGD-4, IG97-147, IG97-132, IG97-158, IGKMFD-1.	Drought susceptible	Injury Index	more
8	IG3108, IG97-245, IG97-144, IBANG-D-1, IG97-152, IG97-151, IG97-189, IG97-132, IG97-218, IG97-147.	Drought susceptible	OA	Low
9	IG97-244, IG97-121, IG97-152, IG97-24, IG95-30, IG97-241, IG95-25, IG97-114.	Drought susceptible	MDA	More
10	IG97-121, IG97-144, IG97-118, IG97-152, IG97-244,I GKMD-10, IG95-30, IG95-25, IG97-114.	Drought susceptible	P5CS	less
11	IG97-245, IG97-144, IG97-152, IG97-132.	Drought susceptible	POD	less
12	IG3108, IG97-121, IG97-144, IG97-151, IG95-30.	Drought susceptible	SOD	More decrease

more susceptible to water stress. Similarly a group of 9 accessions revealed low injury index indicated that these accessions are better tolerant to water stress. However, 11 accessions revealed the moderate injury index value between higher and lower injury index groups. Present findings were also supported by the findings of Shafqate and Azam, (2006) where screening of wheat genotypes under salt and water stress conditions where performed on cell membrane stability (CMS) technique. They concluded that salt tolerant and water tolerant varieties of wheat suffer less membrane injury than those of susceptible genotypes.

Superoxide dismutase (SOD):

Looking at the level of H_2O_2 metabolism, it appears that the components of this metabolism can be directly correlated with the magnitude of water stress as well as with water stress tolerance. Thus SOD, which is responsible for dismutating superoxide to H_2O_2 showed declining trend under water stress. This situation may favour accumulation of O^-_2 under water stress and contribute to membrane damage (Dhindsa and Motowe, 1981). They have reported the decline in SOD activity in a drought sensitive mosses. In the present study among 30 *Dichanthium* accessions under water stress, SOD activity decreased in 27 accessions with respect to their control, whereas 3 accessions namely IG97-118, IGKMFD-1 and IGTGD-4 indicated increase in SOD activity from their respective control. However, in IG9-118 increase in SOD activity was non-significant. The superoxide dismutase (SOD) is the key enzyme in active oxygen scavenger system because it catalyses superoxide free radical dismutation into H_2O_2 and O_2 (Elstner, 1982; Bowler *et al.*, 1992; Scandalios, 1993). However, SOD activity was reported to be increased on onset of water stress but decline as magnitude of water stress increased in bentgrass (Liu and Huang, 2000). In the present study among 27 *Dichanthium* accessions which showed decreased (1.4 to 58.8 %) in SOD activity at severe water stress. Based on decreases in SOD activity at sever water stress, these accessions were categorized into three groups. A group 10 accessions (IGBANG-D-2 IGKMD-10, IG97-24, IG95-25, IG97-247,

IG97-130 IG97-218 IG97-132, IG97-158, IG97-170 showed less decrease in SOD activity form their respective controls ranged from 1.41 to 19.38% with average of 9.9% activity. Six accessions (IG95-30, IG97-121, IGBANG-D-1, IG3108, IG97-144 and IG97-151) showed maximum decrease in SOD activity ranged from 47.19% to 58.36% with average of 52.19% from their controls, where as rest of 11 accessions moderately decreased their SOD activity under sever stress with average of 30.15%. The decrease in SOD activity under sever stress indicated that the scavenger ability in the cells of leaves was inhibited under severe water stress conditions. It is also manifested that the group of accessions which showed less extent of decrease in SOD activity as compared to the group which showed higher decrease in enzyme activity could be better able to tolerate water stress. Liu and Huang (2000) reported similar findings in Bent grass, indicated more decrease of SOD activity in *Penncross* cultivars than L-93, and further concluded that L-93 was better heat tolerant than *Penncross* cultivars. The high SOD activity has been associated with stress tolerance in plants because it neutralizes the reactivity of O_2^- which over produced under stress (Bowler *et al.*, 1992).

In the present study the results indicated that the accessions with lesser decrease in SOD activity sustained a higher active O_2 scavenger ability during water stress, therefore such accessions were defined as more water stress tolerant, hence the selection of grasses using this criteria may help to develop water stress tolerant accessions.

Peroxidase activity:

In general, response of water stress was observed in the increase in peroxidase activity measured using guaiacol as an artificial substrate. Peroxidase catalyzes hydrogen peroxide dependent oxidation of substrate according to the general equation.

$RH_2 + H_2O_2 = 2H_2O+R$

Many researchers have reported increase in peroxidase activity under water stress (Dwivedi *et al.,* 1979; Badiani *et. al.,* 1997; Zhang and Kirkham, 1994). This indicated that formation of large amount of H_2O_2 during water stress. Water stress could increase the accumulation of peroxidase substrate which in turns are scavenger of activated oxygen species (Elstner, 1982; Winston, 1990). The accumulation of these metabolites, could lead to an increase in peroxidase activity in the presence of enhanced levels of H_2O_2 (Zhang and Kirkham, 1994). *Dichanthium* accessions did not show a regular increase or decrease patterns of peroxidase activity under severe water stress level. The results indicated the increased peroxidase activity under water stress in 15 *Dichanthium* accessions and decreased peroxidase activity in 15 accessions stressed condition. Grouping of accessions was made of those accessions where peroxidase activity was increased. A high peroxidase activity (50.4 to197 %) over their control was found in 5 accessions (IG97-170, IG97-118, IGKMG-10, IGBANG-D-2 and IGTGD-4). Least increase in peroxidase activity (4.7 to13.1%) was recorded in 4 accessions (IG97-152, IG97-132, IG97-144, and IG97-245), whereas moderate increase in peroxidase activity was observed in 6 accessions.

Peroxidase constituted a set of enzymes that catalyze the oxidation of substrate by H2O2. The high POD activity would be associated with chlorophyll degradation during leaf senescence, and was likely induced by increased level of superoxide radicals (Kato and Shimizu, 1985). The important role of peroxidase in relation to oxidative tolerance has been reported in many plant species (Gupta *et al.,* 1993; Lee and Lee, 2000; O'Kane *et al.,* 1996). Kuk *et al.* (2003) reported more increased peroxidase activity in cold acclimated (tolerant) lines than those of non acclimated lines. Results indicated in the present study that *Dichanthium* accession with high peroxidase activity have better ability to cope with water stress than those of having low activity.

Pyrroline-5-carboxylate synthetase (P5CS) enzyme activity:

To evaluate whether accumulation of proline as an active process brought about by onset of stress, P5CS activity was determined among *Dichanthium* accessions. In the plants, proline is synthesized in the cytosol and mitochondria from glutamate via two successive reduction reactions catalyzed by Pyrroline 5-carboxylate synthetase and Pyrroline 5-carboxylate reductase enzyme (Hare *et al.*, 1999). Genes encoding these enzymes have cloned in several plants plant species, and expression of P5C was shown to be up regulated by water and osmotic stress (Hare *et al.*, 1999). In addition, there is an evidence that accumulation of product of proline synthesis and catabolism namely glutamine and pyrroline 5-carboxylate (P5C) in plants. Pyrroline 5-carboxylate synthetase activity measured among the 30 *Dichanthium* accession, indicated many fold increase in enzyme activity in 28 accessions and decreased activity in 2 accessions. Highest increase in P5CS activity in 7 accessions (IGKMFD-1, IGBANG-D-1, IGTGD-4, IG97-147, IG97-130, IG97-158 and IG97-241) ranging from 4.32 to 21.68 fold increase over their control with average of 6.77 fold increase, confers them for more production of proline. Another group of 9 accessions confers low activity of enzyme ranged from 1.09 to 1.67 fold with average of 1.39 fold increase whereas 12 accessions exhibited moderate enzyme activity (1.79 to4.12 fold). The findings suggest the increase in activity of P5CS enzyme allows more accumulation of proline amino acid that plays a role in turgor maintenance of the cell. Various studies suggested that increase in P5CS activity confers stress tolerance in plants. Aida *et al.* (2005) suggested that transgenic potato containing cDNA gene of P5CS showed an improved tolerance to salinity. Mattioni *et al.* (1997) studied in wheat, where high P5CS activity confers tolerance in both salt and drought.

Selection of drought tolerant and susceptible accessions:

Based on the performance of 30 *Dichanthium* accessions with various drought responsive traits (RWC, WLR, WP, OP, osmolality, proline content, MDA

Table 29: Water stress tolerant accessions and behaviors of different drought stress related parameters.

S.No	Accessions	Probable category	RWC	WLR	OP	WP	Osmol.	ID	OA	proline	MDA	P5CS	POD	SOD
1	IG97-234	Drought tolerant	+++		+++			+	+++		+			
2	IG97-24	Drought tolerant	+++	+	+++			+	+++		+			+++
3	IG95-30	Drought tolerant	+++	+			+++	+	+++					
4	IG97-247	Drought tolerant	+++		+++			+			+			+++
5	IG97-241	Drought tolerant	+++	+	+++		+++	+				+++		
6	IGBANG-D-2	Drought tolerant		+				+	+++	+++			+++	+++

Accessions took 11 to 13 days to reach average 49 to 50% RWC

Table 30: Water stress susceptible accessions and behaviors of different drought stress related parameters.

S.No	Accessions	Probable category	RWC	WLR	OP	WP	Osmol.	ID	OA	Proline	MDA	P5CS	POD	SOD
1	IG3108	Drought susceptible	+	+++	+	+	+	+++	+	+	+++			+
2	IG97-144	Drought susceptible	+		+			+++	+			+	+	+
3	IG97-121	Drought susceptible			+		+			+	+++	+		+
4	IG97-152	Drought susceptible	+					+	+		+++	+	+	
5	IGTGD-4	Drought susceptible		+++	+	+		+		+				

Accessions took 6 to 8 days to reach average 49 to 50% RWC

+++ **High ,** + **Low**

content, Osmotic adjustment, injury index, peroxidase activity, superoxide dismutase activity, and P5CS activity) accessions were sorted into three probable categories namely probable tolerant accessions, probable susceptible accessions and moderately tolerant accessions with individual parameters (Table 29 and 30). Based on the better performance for different stress parameters following accessions (IG97-234, IG97-24 , IG95-30 , IG97-247 , IG97-241 , IGBANG-D-2) were selected as drought tolerant accessions, where these accessions performed better in 5 to 7 parameters of the 12 studied (Table 29). Six accessions (IG3108 , IG97-144 , IG97-121 , IG97-152 , IGTGD-4) were considered as water stress susceptible kind, where these accessions shown poor performance in 5 to 9 parameters out of 12 (Table 30).

Association of RAPD, ISSR and STS based markers with drought responsive traits:

Polymorphic markers generated from fingerprinting patterns belonging to a narrow gene pool represents independent characters for specific traits (Song *et al.*, 2000). In view of genetic polymorphism observed within the *Dichanthium* accessions using three molecular systems (RAPD, ISSR and STS) an attempt was made to associate easily scorable bands (from DNA marker system) with drought responsive parameters studied. Activity of stress related protective enzymes has been reviewed in length (Smirnoff, 1993; Polle, 1997). Based on the better performance for different stress parameters following accessions (IG97-234, IG97-24 , IG95-30 , IG97-247 , IG97-241 , IGBANG-D-2) were selected as drought tolerant accessions where these accessions performed better in 5 to 7 parameters of the 12 studied. Six accessions (IG3108 , IG97-144 , IG97-121 , IG97-152 , IGTGD-4) were considered as water stress susceptible kind, where these accessions showed poor performance in 5 to 9 parameters out of 12. The association between RAPD bands and trait of interest have been reported by various authors (Buckler, 2002; Nguyen *et al.*, 2004). In the present study a total of 56 RAPD primers were tested for association with eight water stress responsive

traits. Out of 56 primers, 26 primers showed association with one or more stress responsive traits. Among these primers one or more bands were found to be associated with one or more traits. The association was studied at two confidence levels i.e., 99% (0.01) and at 99.9% (0.001). In total 49 RAPD markers were found to be associated with drought tolerance. Out of 49 marker bands, 10 bands showed association with drought tolerant at 99.9% confidence level whereas rest 39 at 99% confidence level (Table 23). Further these bands were compared with their presence or absence in already screened tolerant accessions. And finally 7 bands found a strong association with drought responsive traits. Based on RAPD study, 650bp with primer OPU06, 650bp with OPU04, 400bp with OPR04, 500bp with OPI18 and 550bp with OPAH03 indicated strong association of bands with different drought tolerant traits and these bands were present in drought tolerant accessions and were largely absent in susceptible accessions. Hence these bands could be used in germplasm screening for drought tolerance. These results are supported by similar kind of findings obtained by Mishra and Mandi (2004) using AFLP marker system in drought tolerant tea clones.

Pakniyat *et al.* (1997) screened 39 barley accessions for salt tolerance based on Na^+ ion content and ∂^{13} C and association of 12 AFLP markers were established for salt tolerance in screening of salt tolerant lines. In the present study, ISSR and STS bands were also used for association study with drought responsive traits. In total 13 bands from 7 STS primers had shown association with drought responsive trait, however bands 310 and 200 bps with primer T3F3/T3R3 gave strong association with proline content and was present in drought tolerant accessions. Total 6 ISSR markers had shown association with drought responsive trait in *Dichanthium* accessions, of these, only 400bp band from primer ISSR82 shown strong association with injury index, and it was present in drought tolerant accessions. So being associated with the accessions exhibiting better performance for drought responsive traits, these identified molecular markers could be used in screening of large numbers of *Dichanthium* germplasm for drought tolerance.

Summary

Marvel grass *(Dichanthium annulatum)* is perennial range grass, which can persist and survives under harsh and dry environmental conditions. It is largely apomict in nature with different ploidy levels, however tetraploid are predominates. Of the many species in *Dichanthium* genus, two species namely *D. annulatum* and *D. caricosum* are widely used for forage production in India. Despite the availability of wide genetic diversity and potential for resistance to stress (especially to drought), no attempt has been made using molecular and biochemical markers to characterize the similarity and dissimilarities in *Dichanthium annulatum* accessions and also the association of molecular markers with drought responsive traits. Keeping this in mind the present work was carried out using 30 accessions of *Dichanthium annulatum* with special emphasis on following points:

1. Development of DNA fingerprints of 30 *Dichanthium* accessions using three molecular marker systems, i.e., RAPD, ISSR and STS, and isozyme as biochemical marker.
2. Assessment of genetic relationships and cluster analysis.
3. Water stressed induced changes in water relation parameters i.e., Water potential, Relative water content (RWC) and Osmotic potential (OP).
4. Water stressed induced changes in osmolytes i.e., Total soluble proteins, osmolality, malondialdehyde (MDA) and proline content.
5. Stress induced changes in antioxidant enzyme (peroxidase and superoxide dismutase) and metabolic enzyme (P5CS) systems in plants.
6. Association of specific water stress related parameters with DNA finger prints

Of the 200 random primers, 56 screened and selected primers were used to develop DNA finger prints of 30 *Dichanthium* accessions. In total 510 amplified bands were scored. Fifty six bands were monomorphic and rest were polymorphic accounts for an average 88.51% polymorphism. The average polymorphic information content (0.448) and marker index (3.70) showed suitability to study

genetic relationship and trait association study. In total 17 STS primers were screened for their transferability in *Dichanthium* and 14 primers gave best amplification at 52 ^{0}C annealing temperature, and were used to develop DNA finger prints of *Dichanthium* accession. In total 106 bands were scored, of these, 84 bands were polymorphic and 22 bands were monomorphic. This exhibited an average 80.10% polymorphism. The average PIC (0.426) and MI (2.60) as observed with this marker system provided sufficient transferability and polymorphism to carryout genetic relationship and trait association studies. Five ISSR primers were also used to develop DNA finger prints of *Dichanthium* accessions. In total 52 polymorphic bands was obtained. Higher PIC (0.480) and MI (4.98) values indicated its strong discriminatory power to assess the *Dichanthium* accessions and also provided better option for genetic diversity and trait association studies.

Dendrogram based on RAPD revealed three main clusters separated at 72.61% similarity level. Total 29 accessions were grouped in these clusters, however accession IGBANG-D-2 emerged as single accession in dendrogram and shared only 55.13% similarity with accessions of other clusters. Cluster I and Cluster II together grouped 25 accessions belonging to north Indian region except intermixing of IGKMFD-1 (South India) in cluster II. A group of 4 accessions belonging to south India separated as Cluster III. The genetic diversity and clustering pattern revealed considerable diversity among the *Dichanthium* accession belonging to different region of India. Dendrogram based on STS marker system also revealed three clusters separated at 75% similarity level embodied 29 accessions. Accession IGKMF-D-1(south India) separated out from the rest of the cluster. All the accessions belonging to north India were clustered in cluster I and II. Five out of six accessions in cluster III belonging to south India intermixed with IG97-144 of north India. In total 12 nodes in dendrogram showed boot strap values more than 50% and hence strong clustering of accessions was visualized. Dendrogram based on this marker system fairly separated accessions on the basis of regional distribution of accessions. As RAPD

and STS , ISSR marker system also revealed three major clusters separated at minimum 66/6% similarity level. Majority of accession belonging to north India were clustered in cluster I and II, however intermixing of IGKMFD-1(south India) was also observed in cluster II. Cluster III embodied accession of both regions. In total 12 nodes in dendrogram showed boot strap value more than 50%.

Biochemical marker like isozyme (Peroxides, Esterase, Polyphenol oxidase and Superoxide dismutase) also indicated sufficient polymorphism. Esterase and PPO yielded more polymorphism than those of peroxidase and SOD. In contrast to the molecular markers based assessment, isozyme marker analysis yielded 18 accessions (out of 30) more than 90% similar whereas rest of the 12 exhibited similarity ranging from 67 to 90%. Similar to molecular marker, isozyme also revealed three major clusters at 67% genetic similarity level. Cluster I embodied all the accessions belonging to north India whereas cluster II and III embodied accessions belonging to both the regions. All six south Indian accessions showed wide variability among themselves as well as to the accessions of north central India. Though these accessions were clustered in two different clusters but centered to one part of the phenogram indicating some level of closeness making them to cluster close to each other. In total 14 nodes were identified in dendrogram having more than 50% boot strap value. When these four marker systems were compared (polymorphism, polymorphic information content and marker index), ISSR and RAPD markers were observed more efficient in detecting genetic diversity and polymorphism in *Dichanthium* than STS and isozymes.

When these accessions were analyzed for water stress parameters, relative water content invariably decreased under stress condition in all accessions, however the number of days varied (6-13 days) among the accessions to drop their RWC to the average value of 49%. A group of accessions namely IG97-234, IG97-24, IG95-30, IG97-247, IG97-152, IG97-184 and IGKMFD-1 were put into putative tolerant categories as these accessions preformed better and took more

days to drop their RWC. Another group of accessions namely IG3108, IG97-24, IGBANG-D-2, IGKMD-10, IBANG-D-1 took less days to drop their RWC to average stressed level, hence categorized as drought susceptible. Remaining accessions were considered as moderate drought tolerant type. Water potential decreased with progress of water stress and minimum drop was observed in IG97-24, IG97-184, IG97-218, IGBANG-D-2 accessions and maximum in IG3108, IGTGD-4, IGKMD-10, IG97-158, IG97-118 accessions . Rest of the accessions showed moderately decrease in WP.

In order to visualize the rate of water loss (WLR) at various time intervals (2, 4 and 24 hr) an experiment was also performed indicated more WLR in putative susceptible types of accessions like IG3108 and IG97-118. However, it was less in putative stress tolerant accessions namely IG97-234, IG97-24, IG95-30, IG97-241, IG95-25, IG97-147, IG97-132, IG97-130, IG97-144). Rest of the accessions showed moderate WLR. Under water stress osmolality among the accession was increased uniformly form their corresponding controls. Accessions based on osmolality were categories into three groups. A group of putative tolerant accessions (IG95-30, IGKMFD-1, IG97-241 IGBANG-D-2, IG95-114) showed major increase in osmolality, whereas a group of accessions (IG3108, IG97-121 IG97-245, IBANG-D-1, IG97-130) showed comparatively less increase in osmolality.

Osmotic potential under water stressed plants decreased significantly from their corresponding controls among all the accessions. Maximum decreased (more negative) was observed in a group of accessions (i.e., IG97-234, IG97-24, IG97-247, IG97-241, IG95-114, IG97-151) and these accessions were put into putative tolerant group whereas a group of accessions (IG3108, IG97-121 IG97-244, IG97-245, IG97-144, IGTGD-4, IBANG-D-1) which showed less decrease in OP were put into putative susceptible category.

Different osmoprotectants were measured in 30 accessions of *Dichanthium* to decipher the role of these osmoprotectant in providing the tolerance against drought. Water stress leads to uniform increase in proline content in all the accessions. The level of proline increased was observed many folds. Major increase of proline content was observed in IGBANG-D-2, IG97-147, IGKMFD-1, IGKMD-10, IG97-130, IG97-118 whereas minimum increase was recorded in IG3108, IG97-121, IGTGD-4, IG97-192, IG97-247, IG97-241, IG97-170 accessions. Total soluble protein increased among the accession under water stress, however extent of increase varied among the accessions. A group of accessions (IG3108, IG97-121, IGBANG-D-1, IG97-192, IG95-30, IG97-234, and IG97-151) exhibited maximum increase of protein content whereas minimum by 7 accessions. Rest of the accessions showed moderate increase in protein content. The level of malondialdehyde (MDA) content, which generally described the extent of lipid peroxidation, changed with water stress condition of the plants. High level of MDA was observed in IG97-244, IG97-121, IG97-152, IG97-24, IG95-30, IG97-241, IG95-25, IG97-114 indicated their drought susceptible nature whereas a group of 7 accessions namely IG97-234, IG97-247, IG97-192, IG97-189, IG97-244, IG97-144 and IGTGD-4 exhibited less MDA under stress, hence these accessions were put into putative tolerant category.

Osmotic adjustment (OA) capacity of plants is one of the crucial and important parameters which provide highly significant behavior of the plant in drought conditions was also estimated in 30 accessions of *Dichanthium*. High OA was recorded in accessions IG97-234, IG97-24, IG95-30, IGKMFD-1, IG97-241, IGBANG-D-2, IG95-114, IG97-233 and IGTGD-4 and they were put into putative tolerant category whereas accessions showing less OA like IG3108, IG97-245, IG97-144, IBANG-D-1, IG97-152, IG97-151, IG97-189, IG97-132, IG97-218 and IG97-147 were put into susceptible category. Cell membrane injury which corresponds to electrolyte leakage as injury index was observed high in a group of 10 accessions (IG3108, IG97-144, IG97-118, IG97-152,IG97-151, IGTGD-4, IG97-147, IG97-132, IG97-158, IGKMFD-1 and these accessions

and were put into putative susceptible category whereas 9 accessions namely IG97-234, IG97-24, IG95-30,IG97-192, IG97-247, IG97-241, IGBANG-D-2, IGKMD-10, IGBANG-D-1 showing less injury index were put into putative tolerant category.

The proline synthesizing enzyme like P5CS activity measured among the accessions under both stressed and non stressed conditions indicated that accessions IGKMFD-1, IG97-241, IG97-147, IG97-158, IG97-130, IGTGD-4, IGBANG-D-1 showed higher activity and some of the accessions like IG97-121, IG97-144, IG97-118, IG97-152, IG97-244,I GKMD-10, IG95-30, IG95-25, IG97-114 showed low activity. The results further corroborate the variations in accumulation of proline under stress in different accessions.

Antioxidant enzymes which usually provide tolerance to the plants under abiotic stress like drought was also estimated in *Dichanthium* accessions and results indicated that antioxidant system played an important role in providing the stress tolerance in *Dichanthium* as in other crops. A group of 5 accessions (IGBANG-D-2, IGTGD-4, IGKMD-10, IG97-118, IG97-170) showed high peroxidase activity, similarly a group of 4 accessions (IG97-245, IG97-144, IG97-152, IG97-132) showed less peroxidase activity hence these groups of accessions were put into putative tolerant and susceptible categories. Superoxide dismutase activity decreased in all the accession under water stress condition at the highest level of drought stress. At moderate level of stress, a group of 10 accessions (IG97-24, IG97-247, IG95-25, IG97-170, IG97-158, IG97-132, IG97-18, IG97-130, IGBANG-D-2, IGKMD-10) showed higher enzyme activity whereas a group of 5 accessions (IG3108, IG97-121, IG97-144, IG97-151,IG95-30) showed less enzyme activity, therefore these group of accessions were put into tolerant and susceptible categories respectively.

Based on the performance of 30 *Dichanthium* accessions with various drought responsive traits (RWC, WLR, WP, OP, osmolality, proline content,

MDA content, osmotic adjustment, injury index, peroxidase activity, superoxide dismutase activity, and P5CS activity), these accessions were categorized into three probable categories. Based on the better performance for different stress parameters accessions IG97-234, IG97-24 , IG95-30 , IG97-247 , IG97-241 and IGBANG-D-2 were selected as drought tolerant as they performed better in 5 to 7 parameters of the 12 drought responsive traits studied Similarly, 6 accessions namely IG3108, IG97-144, IG97-121, IG97-152, IGTGD-4 were considered as water stress susceptible as these accessions showed poor performance in 5 to 9 parameters.

When drought stress related parameters and markers data were correlated, a total of 49 RAPD markers were found to be associated drought tolerance form 26 RAPD primers, however out of 49 marker bands 10 bands showed association with drought tolerant at 99.9% confidence level whereas rest 39 at 99% confidence level. Further when these bands were compared with their presence or absence in already screened tolerant accessions, 7 bands were showed a strong association with drought responsive traits. These bands were present in drought tolerant accessions and absent in susceptible accessions. Hence, these bands could be used in germplasm screening for drought tolerance. Along with RAPD bands, associations of ISSR and STS markers with some of drought responsive traits were also attempted. In total 13 bands from 7 STS primers shown association with drought responsive trait. Bands of 310 and 200 bps with primer T3F3/T3R3 gave strong association with proline content and were present in drought tolerant accessions. In total 6 ISSR markers showed association with drought responsive trait in *Dichanthium* accessions. Of these, only 400 bp band from primer ISSR82 shown strong association with injury index, and it was present in drought tolerant accessions. Therefore, the markers showing association with drought responsive traits can be used in screening of large numbers *Dichanthium* germplasm for drought tolerance.

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